Haemolytic virulence factors in Photorhabdus luminescens strain W14

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Abstract

Photorhabdus are potent entomopathogens that enter insect hosts passively via the symbiotic nematode partner. *Photorhabdus* are released directly into the haemocoel and must overcome the insect immune defence to establish infection. *Photorhabdus* are able to grow unrestricted in the insect haemocoel hence they must actively overcome the immune competent cells (haemocytes) that act rapidly to physically entrap invading microorganisms. *In vitro* haemocyte monolayer assays confirmed that *P. luminescens* W14 cell-free supernatant contain multiple toxins and characterised the *P. luminescens* W14-induces haemocyte death, haemocyte actin rearrangements and inhibition of phagocytosis. Similar haemocyte mortality and actin rearrangements were observed for entomopathogen *P. temperata* K122 supernatant treatment after prolonged incubations, while none of these phenotypes were observed after supernatant treatment of non-pathogenic *E. coli*. *Photorhabdus* W14 and K122 cells were able to resist phagocytosis *in vitro* while only W14 cells and supernatant suppressed haemocyte phagocytosis of *E. coli*. Collectively, data suggest anti-haemocyte toxins are exclusive to the entomopathogens used in these studies and that W14 and K122 are likely to have distinct anti-phagocytic strategies during insect infections. Candidate virulence factors: Toxin complex (Tc) toxins, makes caterpillars floppy (Mcf) and a putative lipase (Pdl) were tested for anti-haemocyte toxicity. *In vitro* studies with *P. luminescens* W14 *tc* knockouts showed *tcc*$^-$ displayed reduced cytotoxicity; *tcd*$^-$ caused lower percentage of plasmatocyte with actin rearrangement relative to wildtype and *tcb*$^-$ caused fewer granulocyte actin rearrangement and reduced phagocytosis inhibition, however, *E. coli* expressed Tcb alone is not sufficient to cause these phenotypes. *P. luminescens* W14 Mcf expressed in *E. coli* caused apoptotic-like death of haemocytes *in vitro* while *P. temperata* K122 *mcf*$^-$ displayed an increased LT_{50} relative to wildtype. *P. luminescens* W14 Pdl expressed in *E. coli* lysed sheep red blood cells but lacked toxicity against *M. sexta* haemocytes, Pdl could be a component in the annular haemolytic phenotype of wildtype. The present studies revealed the presence of multiple virulence factors their effects on the haemocytes of a susceptible host *M. sexta*. The potential roles of these candidate toxins are discussed.

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To my family
To Stuart

Thank you for giving me everything
Thank you for believing and understanding

****

My thanks go to everyone with whom I share fond memories of
tea & donuts in the office, indulging meals out or in,
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Abbreviations

°C	degree celsius
BH3	bcl-homology 3
CFU	colony forming units
D.I.C.	differential interference contrast
DNA	deoxyribonucleic acid
FITC	fluorescein isothiocyanate
g	acceleration due to gravity
g	grams
GIM	Grace's insect media
GFP	green fluorescence protein
Gr	granulocytes
h	hours
HPs	hyperphagocytes
K122	*P. temperata* subsp. *temperata* strain K122
l	litre
LB	Luria Bertani
kDa	kilodaltons
µ	micro
m	milli
M	molar
mAb	monoclonal antibody
Mcf	makes caterpillar floppy
min.	minutes
MLST	Multi-Locus Sequence Typing
nm	nanometres
PAIs	pathogenicity islands
PBS	phosphate-buffered saline
Pdl	*Photorhabdus* diacylglycerol lipase-like
PFA	paraformaldehyde
Pl	plasmatocytes
PP3	Proteose Peptone No.3
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
s	seconds
S.D.	standard deviations
SDS	sodium dodecyl sulphate
Tc	toxin complex
TE	Tris EDTA buffer
THC	total haemocyte count
TRITC	tetramethylrodamine isothiocyanate
TTO1	*P. luminescens* subsp. *laumondii* strain TTO1
vol	volume
W14	*P. luminescens* subsp. *ackhurstii* strain W14

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Chapter 1 Introduction

Haemolytic virulence factors in *Photorhabdus luminescens* W14

Photorhabdus is a potent pathogen of invertebrates and an ideal model organism for studying bacterial infection of insects (Silva *et al.*, 2002). This thesis investigates candidate virulence factors responsible for combating insect immune competent cells (haemocytes) using the larvae of the Lepidopteran, *Manduca sexta.* In this introductory chapter I will describe the entomopathogenic bacteria *Photorhabdus* and outline the state of knowledge on insect immunity and bacterial pathogenicity.

1.1 *Photorhabdus* and *Xenorhabdus*

Photorhabdus are Gram-negative entomopathogenic bacteria belonging to the *Enterobacteriaceae* family (Ehlers *et al.*, 1988). *Photorhabdus* bacteria were first isolated by Poinar *et al.* in 1977 being characterised by their characteristic ability to produce light (bioluminescence) and by their symbiotic association with *Heterorhabditidea* nematodes. They were initially classified as '*Xenorhabdus*' *luminescens* by Thomas and Poinar (1979) and later accommodated into a new genus *Photorhabdus* based on phenotypic characteristics and DNA relatedness to *Xenorhabdus* by Boemare *et al.* (1993). Three *Photorhabdus* species have since been identified; *P. luminescens*, *P. temperata* which are nematode symbionts and *P. asymbiotica* consisting of human clinical isolates (Fischer-Le Saux *et al.*, 1999).

Photorhabdus associate with *Heterorhabditis* nematodes and *Xenorhabdus* associate with *Steinernema* nematodes (Liu *et al.*, 2001), the coupling between these bacteria and their symbiotic nematodes is highly stringent (Liu *et al.*, 2001; Szallas *et al.*, 1997; Putz *et al.*, 1990; Akhurst, 1983). Contrary to the strict symbiotic partnerships, the target host range for these bacteria-nematode complexes is less specific. Pathogenicity has been documented against several insect orders including Lepidoptera, Coleoptera, Diptera and Orthoptera.

1.1.1 *Photorhabdus* phylogeny

Photorhabdus and *Xenorhabdus* were defined as separate genera using 16s rRNA sequence phylogenetic analysis by Boemare *et al.* (1993), both genera were classified as members of Enterobacteriaceae by the same method by Suzuki *et al.* (1996). Such analysis was also used to designate different subspecies (Fischer-Le Saux *et al.*, 1999; Akhurst and Boemare, 1988). It is generally accepted that bacteria and nematode form monoxenic association and their phylogenetic relatedness reflect nematode association and geographical origin (Liu *et al.*, 2001; Bonifassi *et al.*, 1999; Ehlers and Niemann, 1998; Fischer-Le Saux *et al.*, 1998; Liu *et al.*, 1997; Szallas *et al.*, 1997; Putz *et al.*, 1990). The emergence of clinical isolates has prompted further studies using 16s rRNA sequence based phylogeny, the findings placed the Australia and U.S.A. clinical isolates in different positions on a phylogenetic tree (Peel *et al.*, 1999; Akhurst *et al.*, 1996) which conflict the recent Multi-Locus Sequence Typing (MLST) analysis placing all clinical isolates into a single group (Marokhazi *et al.*, 2003). Identification of virulence factors, understanding of molecular mechanisms in pathogenicity and subsequent studies in their lineage throughout the strains may shed light on the unresolved phylogeny questions.

Significant interest in the evolutionary relationship between vertebrate and invertebrate bacterial pathogens has led to the whole genome sequence of a U.S.A. clinical isolate *P. asymbiotica* ATCC43949 (in progress at Sanger, Cambridge, U.K.). Valuable information can be gained from comparisons between the genomes of non-pathogenic bacteria (such as the laboratory strain *E. coli* K-2) and numerous established and emerging human pathogens. The *P. asymbiotica* genome will be useful for comparisons with sample sequence data of the nematode associated *P. luminescens* subsp. *akhurstii* strain W14 (ffrench-Constant *et al.*, 2000) and *P. temperata* subsp. *temperata* strain K122 (ffrench-Constant, unpublished), as well as the full genome sequence of *P. luminescens* subsp. *laumondii* TTO1 (Duchaud *et al.*, 2003).

1.1.2 *Photorhabdus* life cycle

Photorhabdus luminescens are carried in the intestine of free-living infective juvenile *Heterorhabditis* nematodes (Ciche and Ensign, 2003). These dauer juveniles do not feed and are resistant to environmental stresses that seek out insect prey in the soil (Forst and Clarke, 2002). Once in contact with a potential host the nematode penetrates the insect via natural openings of respiratory spiracles, mouth or anus (Forst *et al.*, 1997). Additionally, *Heterorhabditis* have their tooth-like appendage near the mouth suggesting possible penetration of the cuticle entering the haemocoel directly (Forst and Clarke, 2002). Upon access to the insect haemocoel, unknown signal(s) trigger nematodes to regurgitate *Photorhabdus* directly into the haemolymph (Ciche and Ensign, 2003). Here *Photorhabdus* replicates and release factors to enable nematode growth and development, as well as virulence factors, which lead to eventual death of the insect (Daborn *et al.*, 2001).

Photorhabdus grow within the insect cadaver with little restriction (Daborn *et al.*, 2001) and produce a wide range of antibiotics to maintain a monoxenic bacterial population (Akhurst, 1982) while providing a rich food source for their symbiotic nematode partner. The *Heterorhabditis* nematodes feed on the bacteria and degrade insect tissue ensuring development into mature self-fertilising hermaphrodites (Forst and Clarke, 2002). The depletion of nutrients over time or other unknown factor(s) provide a signal to switch nematode development to halt at the infective juvenile form. At this point, *Photorhabdus* are repackaged and the nematodes migrate from the spent cadaver in search for new insect host (Forst and Clarke, 2002). The *Photorhabdus*-carrying dauer juveniles are released from the spent insect carcass and the cycle starts over.

1.1.3 *Photorhabdus* symbiosis

Photorhabdus lead an unusual tri-partite lifecycle inhabiting diverse environments such as nematode intestine and insect haemocoel and hence must sense the changing environmental cues and adapt accordingly. There are common molecular mechanisms in bacteria-host interactions in pathogenicity and symbiosis (Hentschel *et al.*, 2000), despite the obvious difference in the hosts' fate in pathogenic and

symbiotic relationships. It is difficult to distinguish between virulence and symbiosis factors in bacteria that act both as a pathogen and a symbiont. In *Photorhabdus*, this problem is complicated by the obligate bacteria-nematode association in nature, that is the *Heterorhabditis* nematodes only develop into infective juveniles in the presence of the correct *Photorhabdus* strain within an insect (Han and Ehlers, 2000). Therefore, many symbiosis factors play important roles in pathogenesis in nature since they are crucial to nematode survival which in turn is essential for bacteria viability as free-living *Photorhabdus* have not been found (Forst *et al.*, 1997). The present *Photorhabdus-M. sexta* infection model will help identify virulence factors with direct toxicity against *M. sexta*, since injection of laboratory grown *P. luminescens* W14 without the nematode partner cause larval death.

Several loci in *Photorhabdus* have been isolated and found to be important for maintaining bacteria-nematode symbiosis, these include regulatory factors reported to have influence on *Photorhabdus*' ability to support nematode growth and development. Forst & Clarke (2003) provides a detailed review of the bacteria-nematode symbiosis and their involvement in the various stages of infection. An example of putative 'symbiosis' factors include crystal proteins of unknown function encoded by *cipA* and *cipB* which were shown not to be necessary for pathogenicity against *M. sexta* (Bintrim and Ensign, 1998).

Bioluminescence emission is a unique characteristic of *Photorhabdus* that was proposed to protect nematode from insect host immunity (ffrench-Constant *et al.*, 2003; Forst and Clarke, 2002). Luciferase is encoded within the *lux* operon and responsible for the bioluminescence visible in late infection, previously hypothesised to attract or repel insects (Forst and Nealson, 1996), or as an oxygen-scavenging pathway to protect *Photorhabdus* itself and the nematode from reactive oxygen species (ROS) damage by preventing the production of damaging ROS in the insect cadaver (ffrench-Constant *et al.*, 2003; Forst and Clarke, 2002). The *lux* operon was proposed to be essential for bacterial viability due to the absence of recoverable *lux* mutants from saturated transposon mutagenesis of *P. luminescens* subsp. *temperata* strain K122 (ffrench-Constant *et al.*, 2003). However, the biological significance of bioluminescence in *Photorhabdus* remains obscure and open to speculation.

The production of anti-microbial peptides is another characteristic of *Photorhabdus*. There is a range of small molecule antibiotics (Couche and Gregson, 1987; Akhurst, 1982); (Derzelle *et al.*, 2002; Hu and Webster, 2000; Bondi *et al.*, 1999; Hu *et al.*, 1997; Li *et al.*, 1995; Li and Meighen, 1992), phage tail-like bacteriocins (Thaler *et al.*, 1995; Poinar *et al.*, 1989) and some are enzymatic as predicted by sequence homology (Sharma *et al.*, 2002; Ciche *et al.*, 2001; Chen *et al.*, 1996). The presence of these anti-microbial peptides is thought to prevent the growth of other microorganisms and maintain a mono-culture within the insect cadaver, ultimately optimising conditions for the growth of the nematode partner (ffrench-Constant *et al.*, 2003).

1.1.4 *Photorhabdus* virulence

Upon entry into their host, the utmost important task for the pathogen is to kill or disable host cells that would otherwise impede its persistence. Bacteria generally do so by actively targeting molecular components of the host immune system to combat, suppress or neutralise immune responses, while other pathogens employ strategies to prevent detection and subsequent initiation of immune responses (Finlay and Falkow, 1997). All molecules that contribute to a pathogen's persistence and survival are described as virulence factors, however, virulence is a complex process and that roles of these factors vary wildly. Wassenaar *et al.* (2001) proposed a refined descriptive nomenclature system to define virulence factors that would lead to more precise annotation of putative virulence factors in aid of furthering our understanding of pathogenicity.

Much recent research efforts concentrate on the identification and characterisation of *Photorhabdus* toxins (detailed reviews in ffrench-Constant *et al.*, 2003; Dowds and Peters, 2002; Forst *et al.*, 1997; Forst and Nealson, 1996). Methods used in identifying potential virulence factors in *Photorhabdus* include screening plasmid and cosmid libraries in *E. coli* for acquired phenotypes or screening random mutant banks of the pathogens for attenuated virulence. Candidate virulence genes identified by sequence homology may be tested by heterogeneous expression or by directed gene knockout in the pathogen itself. Mutant banks can be created by

chemical or transposon mutagenesis and more specific genetic manipulation introduces selectable plasmids by electroporation or conjugation (Forst and Nealson, 1996). However, established techniques for DNA transfer into *Photorhabdus* are limited by strain specificity, therefore the molecular approaches for genetic modification in *Photorhabdus* need further development (Forst *et al.*, 1997).

P. luminescens subsp. *Akhurstii* strain W14 sample sequence analysis by ffrench-Constant *et al.* (2000) compared available *P. luminescens* W14 genomic sequence to a non-pathogenic *E. coli* K12 and identified homologous genes (house keeping) and genes unique to *P. luminescens* W14 (putative pathogenic or symbiotic factors). Waterfield *et al.* (2002) studied putative pathogenicity islands (PAIs) encoding known toxin homologues likely to have role(s) in pathogenicity. Various PAIs identified contained evidence for DNA mobility and rearrangement (Waterfield *et al.*, 2002a). The presence of homologues and orthologues to known anti-phagocyte toxins of vertebrate pathogens within these PAIs suggests *P. luminescens* is likely to share common strategy with, and recent common ancestor to, pathogens such as *Yersiniae* (Fallman *et al.*, 2002; Waterfield *et al.*, 2002a). It is suggested that the acquisition of these factors from the 'pathosphere' may have enabled some *Photorhabdus* strains to colonise human wounds and hence the emergence of clinical isolates (Waterfield *et al.*, 2002a). Several *P. luminescens* W14 toxins of interest to this project are discussed in detail.

1.1.4.1 Toxin complex (Tc) toxins

The best-characterised *Photorhabdus* toxins are the high molecular weight toxin complex (Tc) toxins that were originally identified in *P. luminescens* W14. These orally toxic protein complexes consist of components between 30 to 200 kDa (Bowen *et al.*, 1998). There are numerous *tc* loci throughout the *P. luminescens* W14 genome and some are found in putative pathogenicity islands (Waterfield *et al.*, 2002a). The orally toxic properties reside with toxin complexes Tca and Tcd encoded by *tcaA1*, *tcaB1*, *tcaC1* and *tcdA1, tcdB1*, respectively while the other *tc* loci have minor contributions (Waterfield *et al.*, 2002a; Waterfield *et al.*, 2001a; Bowen and Ensign, 1998).

Histopathological studies revealed that feeding TcA to *M. sexta* larvae causes blebbing of mid-gut epithelium cells and their eventual disintegration (Blackburn *et al.*, 1998), which is comparable to similar phenotypes observed in wildtype W14 infection via injections (Silva *et al.*, 2002), however, the precise role of the Tc components in pathogenicity remains unclear. The apparent ability of Tc toxins to work from both luminal and haemocoel sides to cause similar phenotypes prompts speculation that alternate toxin targets such as haemocytes (Bowen *et al.*, 1998) which warrants further investigation.

1.1.4.2 Makes caterpillars floppy (Mcf) toxin

The *P. luminescens* W14 Mcf (Makes Caterpillar Floppy) is a candidate virulence factor. The clone was found by screening of a *P. luminescens* W14 cosmid library in *E. coli* by injection into *M. sexta* larvae and selection for insect death (Daborn *et al.*, 2002). The open reading frame was identified by transposon mutagenesis of the cosmid and screening again for larval death by injection. Its predicted amino-acid sequence contains carboxy-terminal homology to RTX-like export domain, a central *Clostridium* cytotoxin B translocation domain and a consensus BH3-like motif at the amino-terminus (Daborn *et al.*, 2002).

Exogenous expression of *P. luminescens* W14 Mcf in *E. coli* is sufficient to kill *M. sexta* larvae, histopathology of the infected larvae show disintegration of the midgut with epithelial cell blebbing away indicative of apoptosis similar to that observed in wildtype *P. luminescens* W14 infection (Daborn *et al.*, 2002). Further, a cytosolic fraction of recombinant Mcf causes membrane blebbing of *M. sexta* haemocytes *in vitro*. The importance of Mcf as a toxin is supported by its presence in all *Photorhabdus* strains examined to date (Daborn *et al.*, 2002). Mcf also displays toxicity against several mammalian cell-lines and causes caspase-3 dependent apoptosis with evidence for a functional BH3 domain (Dowling *et al.*, 2004). However, the precise role of Mcf in *Photorhabdus* virulence is unclear and its *in vivo* expression and mode of action are unknown. More specifically the involvement of BH3 domain in cytotoxicity and its relevance in bacterial virulence warrant further investigation.

1.1.4.3 Lipases and proteases

Enzymatic functions such as bacterial lipases and proteases have often been attributed to roles in virulence (Miyoshi and Shinoda, 2000; Titball, 1998). Secreted factors in the cell-free culture supernatant of *P. temperata* subsp. *temperata* strain K122 were found to be toxic against *G. mellonella*, and a lipase was identified that accounts for part of the supernatant toxicity (Clarke and Dowds, 1995). Jarosz (1998) revealed a secreted protease from *H. bacteriophora-P. luminescens* complex that actively degrades *G. mellonella* lysozyme and cecropin-like immune molecules. While Caldas *et al.* (2002) described a *X. nematophilus* protease (protease II) able to destroy antibacterial molecule cecropin A in the insect haemolymph. Bowen *et al.* (1998, 2000) isolated an alkaline zinc-metalloprotease (PrtA) from *P. luminescens*, the purified protein displays toxicity against mammalian cells (Bowen *et al.*, 2003) but not insect haemocytes (unpublished work). The *in vivo* expression pattern of *prtA* in *P. luminescens* W14 infected *M. sexta* suggests a role in biodegradation (Daborn *et al.*, 2001), although pathogenicity has not been ruled out. Both lipases and protease have potential to disrupt host cell membrane integrity hence represent candidate virulence factors.

1.1.5 Role of *Photorhabdus*-insect models

Photorhabdus isolates have been identified in human wounds (Peel *et al.*, 1999; Farmer *et al.*, 1989) and are hence described as an emerging human pathogen (Gerrard *et al.*, 2003). These clinical strains and their closely related nematode-associated isolates could serve as models for bacterial pathogenicity research (Daborn *et al.*, 2001; Waterfield *et al.*, 2001b). Although many mammals have been established as model hosts for bacterial infections (Lengeling *et al.*, 2001), alternatives such as plants and insects are being explored and findings reveal some conserved toxin mechanisms in different hosts (Mahajan-Miklos *et al.*, 2000). Insects and other invertebrates have advantages over mammalian model hosts such as low cost, genetic and physiological malleability and fewer ethical considerations (Silva *et al.*, 2002). Currently utilised insect hosts include *Caenorhabditis elegans* (Aballay and Ausubel, 2001; Gallagher and Manoil, 2001; Garsin *et al.*, 2001; O'Quinn *et al.*, 2001), *Drosophila melanogaster* (Wu *et al.*, 2001, D'Argenio *et al.*,

2001; De Gregorio *et al.*, 2001), *Bombyx mori* (Kaito *et al.*, 2002) and *M. sexta* (Beckage and Gelman, 2004; Silva *et al.*, 2002; Daborn *et al.*, 2001).

1.1.5.1 *P. luminescens–M. sexta* model

Virulence of a bacterial pathogen may be determined experimentally either by the number of cells required to cause death of a host within a given time or as the time taken to cause death by a set cell number (Notermans and Hoornstra, 2000). However, for potent pathogens such as *Photorhabdus* with low LD_{50} hence reduction in bacteria count cannot be accurate determined, virulence can be measure by LT_{50} (Forst *et al.*, 1996; ffrench-Constant *et al.*, 2003).

Despite the absolute requirement of specific bacteria-nematode combination to complete the lifecycle in nature, Akhurst (1980) discovered that some *Xenorhabdus* isolates could be grown *in vitro* in laboratory conditions, maintaining their entomopathogenicity without their nematode partner. Thus, *Xenorhabdus* and *Photorhabdus* can be studied independently of the nematode symbiont. For the purpose of dissecting the underlying pathogenic mechanisms, experiments can be conducted by injection of *Photorhabdus* alone into *M. sexta* larvae to mimic their release directly into the insect haemocoel by the nematode.

Most commonly used model host for *Photorhabdus* and *Xenorhabdus* research are lepidopteran larvae namely *G. mellonella, B. mori, Spodoptera* spp. and *M. sexta.* Fifth instar larvae of *M. sexta* is the chosen model host for the present studies, they are relatively more robust than *G. mellonella* larvae against bacterial infection (Horohov and Dunn, 1983) and have been shown to be susceptible to *Photorhabdus* infection (Bowen *et al.*, 1998). Additionally the larvae are relatively large in size and provide a plentiful source of haemolymph. Despite the lack of genomic data available for *M. sexta*, it has been in use to investigate insect immunity broadly hence its immune responses are relatively well documented for an invertebrate (Tunaz *et al.*, 2003; Zhu *et al.*, 2003; Yu *et al.*, 2002).

1.2 Insect immunity

Insects inhabit environments where microorganisms thrive; insects must have effective means of avoiding and combating infections. Once passed the physical barriers of the outer exoskeleton, peritrophic membrane or the tracheal linings, the intruders must contend with the insect's immune system (Hallman *et al.*, 2001; Trenczek, 1998). The insect immune systems can be divided into cellular and humoral responses; cellular responses act to physically contain the non-self material while humoral responses involve soluble anti-microbial components in the haemolymph (Lavine and Strand, 2002; Trenczek, 1998).

Despite the relative simplicity of the insect immune system, it shares similarities with the vertebrate innate immunity (Williams, 2001; Vilmos and Kurucz, 1998). Innate immunity is an ancient form of host anti-microbial response that is shared amongst invertebrates, plants and vertebrates (Medzhitov and Janeway, 1998b). Somatically encoded factors recognise conserved microbial structures and activate immediate responses involving cellular and humoral factors, which are independent of previous exposure to a pathogen (Lavine and Strand, 2002). In vertebrates, innate immune responses play an important role in activating acquired immunity (Janeway and Medzhitov, 2002). Acquired immunity involves somatic gene rearrangements to generate lymphocytes expressing random and unique antigen receptors and kill microorganism by generating antigen-specific lymphocyte and antibodies, memory against the particular antigen give rise to a more efficient response against secondary encounter (Mulnix and Dunn, 1995).

The current working model for anti-bacterial infection in insects describes a multi-phasic strategy involving intricate coordination between humoral and cellular responses (summarised by Mulnix and Dunn, 1995; Ratcliffe, 1993). Once the bacteria enter into the insect, epidermal injury triggers activation of haemocytes which initiates the coagulation of haemolymph to form a fibrous mesh to trap bacteria and to cover the wound (Mulnix and Dunn, 1995). A putative serine proteinase in *M. sexta* haemolymph scolexin is induced by infection but found not to be bacteriocidal and thought to play a regulatory role (Finnerty *et al.*, 1999). Secondly, bacteria are removed from the haemolymph via phagocytosis by

circulating haemocytes (Lavine and Strand, 2002). If bacteria exceed the number that can be removed by phagocytosis alone, haemocytes aggregate to form nodules which are stabilised by melanisation (Lavine and Strand, 2002). Up to 90% of 1 x 10^6 bacteria was cleared within 30 minutes post-injection in *M. sexta* (Horohov and Dunn, 1983). The number of haemocytes would deplete dramatically during this time and then antimicrobial peptides are synthesised constitute the third phase; lysis of bacteria by antimicrobials in the haemolymph and lysozome to degrade bacterial debris (Mulnix and Dunn, 1995; Ratcliffe, 1993). Insect immunology research has revealed common groups of antimicrobials and their modes of action and regulation of synthesis (detailed reviews in Otvos, 2000; Brey and Hultmark, 1998; Boman and Hultmark, 1987). While the humoral responses are investigated at the molecular level, cellular responses remain largely descriptive (Lavine and Strand, 2002).

1.2.1 Recognition

Recognition of non-self is requisite for the initiation of appropriate immune responses (Hallman *et al.*, 2001; Medzhitov and Janeway, 1998a). Pathogen associated molecular patterns (PAMPs) are highly conserved microbial structures unique to pathogens and absent in the hosts (Janeway and Medzhitov, 2002; Trenczek, 1998) such as lipopolysaccharide (LPS) of gram-negative bacteria, peptidoglycan of gram-positive bacteria and β-1,3-glucans and mannans of fungal cell wall. It is these PAMPs by which pattern recognition receptors (PRRs) can detect non-self invaders and initiate downstream immune responses (Medzhitov and Janeway, 1997; Lackie, 1988). Abiotic particle can also be recognised in insects as nonself as determined by physical properties such as surface charge or hydrophobicity (Da Silva *et al.*, 2002; Pech and Strand, 2000).

There are several known insect proteins with affinity for conserved microbial structures and hence potential as pathogen recognition receptors (PRRs; Medzhitov and Janeway, 2000; Soderhall and Cerenius, 1998). These include peptidoglycan binding proteins (PGBPs; Morishima *et al.*, 1997; Dunn, 1986), β-1,3-glucan recognition protein (βGRPs; Ma and Kanost, 2000), LPS- and glucan-binding proteins (LGBPs; Lee *et al.*, 2000) and lectins which are common to both mammals and insects (Vasta *et al.*, 1999). Lectins have specific multivalent capacity and can

bind sugar residues on the pathogen and haemocytes bridging pathogens and phagocytes as an opsinin, haemocyte surface receptors may also be present to bind PAMPs directly although none has been identified in insects to date (Lavine and Strand, 2002).

Several groups of pathogen recognition receptors have been described in *M. sexta* but the signalling pathway components are unknown. Hemolin is a 47 kDa protein with four I-set type immunoglobulin domains belonging to the immunoglobulin superfamily (Sun *et al.*, 2001; Ladendorff and Kanost, 1991). Hemolin binds to haemocyte and bacteria (Sun *et al.*, 2001; Zhao and Kanost, 1996; Ladendorff and Kanost, 1991) and is induced by bacterial challenge (Wang *et al.*, 1995; Ladendorff and Kanost, 1991). Hemolin can bind LPS and lipoteichoic acid (Yu and Kanost, 2002), and cause aggregation of bacterial components and haemocytes suggesting its active role in enhancing cellular responses.

M. sexta peptidoglycan recognition protein (PGRP) is a 19 kDa protein with phage lysozyme-like domain (Yu *et al.*, 2002). PGRP bind to peptidoglycan present on the surface of gram-positive bacterial cell walls and its expression increases with peptidoglycan or bacteria in the haemolymph (Yu and Kanost, 2002). PGRP and peptidoglycan interaction initiates the phenoloxidase cascade, activating many immune pathways (Yoshida *et al.*, 1986) similar to the triggering of PGRP of cytokine and chemokine production in mammalian immunity (Choe *et al.*, 2002; Vilmos and Kurucz, 1998). *M. sexta* β-1,3-glucan recognition protein (βGRP) has high specificity for β-1,3-glycan (Ma and Kanost, 2000) and Gram-negative bacteria-binding proteins (GNBP) have high affinity for LPS of Gram-negative bacteria (Lee *et al.*, 2000).

Four C-type lectins have been cloned from *M. sexta* namely immulectins 1 – 4 (Yu *et al.*, 2002; Yu and Kanost, 2000). Like their mammalian homologues they each have two carbohydrate recognition domains (CRDs) with differing specificities for various sugars (Weis *et al.*, 1998). By binding the terminal monosaccharide residues of the bacterial or fungal cell wall surface components, they initiate immune responses (Weis *et al.*, 1998).

1.2.2 Signalling cascades

The available complete genome sequence of *D. melanogaster* renders it the chosen model to decipher the molecular mechanisms. The use of *Drosophila* microarrays revealed differential gene expression profile induced by different microbes; fungal and gram-positive bacterial components are thought to activate the Toll pathway while gram-negative bacterial components activate the Immune deficiency (Imd) pathway (Ramet *et al.*, 2002; De Gregorio *et al.*, 2001). However, there is redundancy in the binding specificity of the receptors to pathogen-surface protein hence pathways activated can overlap (Yu *et al.*, 2002).

Medzhitov *et al.* (1997) found the human homologues to *Drosophila* Toll, the so-named Toll-like receptors (TLRs). The first TLR was shown to be involved in the production of inflammatory cytokines and co-stimulatory molecules. The cytoplasmic domain of all known TLRs are structural homologous to that of IL-1 receptor family now termed Toll/IL-1 receptor (TIR) domain, while the extracellular domains of these family members are different, where IL-1 receptors have characteristic Ig-like domain, the Toll-like receptors have leucine-rich repeats (Medzhitov and Janeway, 1997). Toll-like receptor ligands are under investigation and have been shown to bind various microorganism components directly, including LPS (Lien *et al.*, 2000; Poltorak *et al.*, 1998), lipoproteins (Takeuchi *et al.*, 2000; Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999), peptidoglycan (Underhill *et al.*, 1999; Yoshimura *et al.*, 1999), bacterial DNA (Hemmi *et al.*, 2000; Ozinsky *et al.*, 2000).

Unlike the mammalian Toll-like receptors, *Drosophila* Toll does not directly interact with either the invading microorganisms or pathogen components, but binds an extracellular molecule Spätzle (Lemaitre *et al.*, 1996). Spätzle is a diffusible molecule, which undergoes proteolytic activation from its inactive form upon microbial invasion (reviewed by (Imler and Hoffmann, 2000; Belvin and Anderson, 1996). It should be noted that the actual pattern recognition molecules in *Drosophila* are yet to be identified, however, the downstream proteolytic cascade components were uncovered to be Gastrulation Defective (Gastr. Def.), Snake and Easter, which are regulated by various serine protease inhibitors (Serpins) such as Necrotic (Nec; (Robertson *et al.*, 2003). The carboxyl-terminal fragment of Spaetzle binds Toll to

transduce the signal through an adaptor protein Tube and a kinase Pelle which phosphorylates Cactus (Xiao *et al.*, 1999; Norris and Manley, 1996; Grosshans *et al.*, 1994). The phosphorylated Cactus is designated for degradation and hence releases transcription factors Dorsal, Dorsal-related immunity factor (Dif) and Relish (Rel) to translocate into the nucleus to modulate anti-microbial peptide production (Han and Ip, 1999).

Pathways involving *immune deficiency* (*imd*) and *immune related deficient* (*ird*) gene products also regulate anti-microbial peptide synthesis through a transcription factor, Relish (Hedengren *et al.*, 1999; Wu and Anderson, 1998). Hedengren *et al.* (1999) showed *relish* mutant flies to have reduced ability to induce anti-microbial peptide production upon bacterial invasion. The activation mechanism of the *imd*-dependent pathway is not yet fully, De Gregorio and colleagues (2001) showed interaction between death domain containing proteins Imd, dFADD and DREDD (caspase-8 homologue). The activation of an IκB kinase signalosome-like complex leads to the cleavage of Relish, the activation process requires a mitogen-activated protein kinase kinase kinase component. The insect mitogen-activated protein kinase (MAPK; (Han *et al.*, 1998) and JAK-STAT (signal transducer and activator of transcription: (Agaisse *et al.*, 2003) have also been analysed for their potential analogous roles in immunity.

The Toll and *imd* transduction pathways are analogous to the well-characterised NF-κB/IκB transduction pathway in mammalian inflammatory response (Imler and Hoffmann, 2000; Ghosh *et al.*, 1998; Belvin and Anderson, 1996). The parallel between the insect and mammalian pathways was consolidated by the discovery of κB-binding sites present in the anti-microbial peptide promoter sequences (Georgel *et al.*, 1995). The equivalent pathways in other insect are not known in such detail, however, many signalling and regulatory pathways of insect immune responses described involved components of proteolytic enzymes to relay cellular signals via a rapid proteolytic conversions (Jiang *et al.*, 2003; Kanost, 1999; Wittwer and Wiesner, 1998). Aside the regulation of antimicrobial peptide synthesis, these cascades are likely to influence cellular responses such as phagocytosis and nodulation (Gillespie *et al.*, 1997).

1.2.3 Phenoloxidase cascade

Phenoloxidase is an oxidoreductase downstream of a rapid activation system that contributes to the immediate immune responses in invertebrates subjected to microbial infection (Ashida and Brey, 1998; Ashida, 1990). Upon recognition of non-self components, the proteolytic cascade within the pro-phenoloxidase activation system (proPO-AS) is initiated to activate pro-phenoloxidase by tightly regulated proteolytic cleavage (Jiang *et al.*, 2003). The active phenoloxidase converts mono- and di-phenols to quinones, which undergo non-enzymatic polymerisation to form insoluble and pigmented melanin easily observed coating encapsulated objects and around healed wounds (Nappi and Vass, 1993). Both melanin and its intermediary compounds have anti-microbial properties (Yu *et al.*, 2003; Nappi and Vass, 1993). Further, activated proPO-AS components gain biological activities to participate in other immune responses such as opsinisation of microorganisms to stimulate phagocytosis (Johansson and Soderhall, 1996).

Both pro-phenoloxidase and phenoloxidase of *M. sexta* have been cloned and purified for enzymatic studies (Jiang *et al.*, 1997; Hall *et al.*, 1995). Pro-phenoloxidase is activated by proteolytic cleavage initiated by detection of microbial components such as LPS, peptidoglycan or β-1,3-glucan by recognition molecules (Yu and Kanost, 2002). Three prophenoloxidase activating proteinases (PAPs) were previously cloned and shown to activate prophenoloxidase by proteolytic cleavage and their transcription is up-regulated in the fat body after bacterial infection (Jiang *et al.*, 2003; Jiang *et al.*, 1998). Zhu *et al.* uncovered several serine proteinases, serine proteinase inhibitors and a serine proteinase homologue (SPH with homology to a human mannan-binding lectin-associated serine proteinase) with increased expression after infection. Also detected was an induced mRNA transcript level of DOPA decarboxylase (DDC), which was previously identified by Hiruma *et al.* (1995) to convert DOPA to dopamine, a substrate to phenoloxidase. DDC mRNA transcription was also induced by bacterial challenge in *Drosophila* (De Gregorio *et al.*, 2001). It was assumed that DDC functions to keep up the supply of precursors for melanisation in response to infection. These humoral regulatory and effector proteins with vital roles in immunity are likely targets for pathogens to suppress or disrupt the insect immunity.

1.2.4 Anti-microbial peptides

The humoral immune responses constitute mainly of immune proteins with anti-microbial properties (Boman, 2003; Otvos, 2000). Upon recognition, proteolytic cascades are immediately activated to initiate localised melanisation and haemolymph coagulation and to induce the synthesis of potent anti-microbials predominantly in the fat body and some haemocytes (Lavine and Strand, 2002). These molecules are secreted and contribute to protection against the invasive microbes in the haemolymph (Ferrandon *et al.*, 1998; Hultmark, 1993). Lysozyme was the first anti-microbial to be discovered and purified from insects (Powning and Davidson, 1976; Mohrig and Messner, 1968). Lysozyme has been cloned and sequenced from various insect orders with the overall structure of the genes conserved. Mulnix and Dunn (1994) cloned the *M. sexta* lysozyme and showed evidence of it to play an important role in initiating and sustaining humoral responses as previously postulated (Dunn *et al.*, 1985). *M. sexta* lysozyme is present in naive larvae haemolymph which is not bactericidal (Horohov and Dunn, 1982), its transcription is elevated most dramatically in the fat-body when exposed to bacterial membrane fragment peptidoglycan (Mulnix and Dunn, 1995). Lysozyme has muramidase activities to hydrolyse peptidoglycan polymer of bacterial cell wall and thought be involved in bacterial killing (Mulnix and Dunn, 1995; Spies *et al.*, 1986). Collectively, constitutive yet low expression of lysozyme is suggested to aid debris clearance from the initial cellular response of phagocytosis, while its elevated presence actively kills bacteria by breaking down the bacterial cell wall and finally releasing fragments of peptidoglycan to trigger anti-bacterial peptide production and thus more lysozyme (Mulnix and Dunn, 1994).

In *Drosophila*, the antimicrobial peptides against fungal and gram-positive invaders are expressed under the Toll pathway control (Lemaitre *et al.*, 1996), while the gram-negative invaders initiate antibacterial peptides expression through *imd* pathway (Lemaitre *et al.*, 1995). Numerous anti-microbial peptides from insects have been described which form four distinct groups (Bulet *et al.*, 1999; Trenczek, 1998; Hoffmann, 1995; Mulnix and Dunn, 1995).

Insect defensins are low molecular weight peptides with homology to those found in vertebrates and plants (Hoffmann and Hetru, 1992). These bear six cysteine residues which interact to form three intra-molecular disulphide bonds to hold a hairpin-like β-sheets or α-helical-β-sheet mixed structures (reviewed in Dimarcq *et al.*, 1998). The insect defensins are typically 4 kDa and potent against gram-positive bacteria are less active towards gram-negatives or fungi (Bulet *et al.*, 1999). Data suggest defensins to form cytoplasmic membrane channels leading to the leakage of divalent cations and hence membrane depolarisation (Ganz and Lehrer, 1994), similar to insect and mammalian defensins. Drosomysins are bigger at 5 kDa, found only in *Drosophila* to date and exclusively active against filamentous fungi via a mechanism yet to be elucidated (Landon *et al.*, 1997; Fehlbaum *et al.*, 1994).

Cecropins and cecropin-like peptides are linear peptides without cysteine and were first found in insects. They contain 15-45 amino acid residues and have a net positive charge. They are active against gram-negative and gram-positive bacteria (Andra *et al.*, 2001). The exact mechanism by which they kill bacteria is not clearly understood, however, peptide-lipid interactions have been shown to lead to membrane permeation implying a role in their activity (Oren and Shai, 1998). These amphipathic α-helical peptides can permeabilise bacterial membrane via either one of the two mechanisms, formation of a transmembrane pore or membrane destruction/solubilization via a "carpet-like" mechanism (Oren and Shai, 1998).

The proline-rich peptides comprise a family of 2-3 kDa with <25% being proline and is without cystiene. They are predominantly active against gram-negative although the mode of action of proline-rich peptides is not understood at present (Trenczek, 1998; Hoffmann *et al.*, 1996). It has been proposed that the proline-rich peptides derive from a common precursor, based upon the high sequence similarity between the known members (Hoffmann *et al.*, 1996).

The glycine-rich family of inducible antibacterial molecules includes several 9 to 30 kDa polypeptides, with 10-22% glycine as content (Trenczek, 1998; Hoffmann *et al.*, 1996). These polypeptides are predominantly active on Gram-negative bacteria with yet uncharacterised mode of action.

Several antibacterial proteins such as cecropin-like proteins, attacin-like proteins, hemolin, M13 and lysozyme have been shown in *M. sexta* (Yu and Kanost, 2000; Spence *et al.*, 1992; Ladendorff and Kanost, 1991; Dickinson *et al.*, 1988). *Photorhabdus* must either prevent the synthesis of antimicrobial peptides or neutralise or destroy them in order to survive and replicate in the haemolymph.

1.2.5 Cellular responses

Microorganisms must penetrate the insect integument, peritrophic membrane or tracheal lining to reach the insect haemocoel. The initial entry of the invaders would activate the formation of a fibrous network which seals the wounding site followed by melanisation as shown in *M. sexta* larvae (Geng and Dunn, 1988). The invaders would then meet the circulating insect haemocytes capable of phagocytosis, nodulation and encapsulation (Yokoo *et al.*, 1995; Geng and Dunn, 1989; Ratcliffe and Rowley, 1979). Haemocytes rely on humoral factors for recognition and initiation and work in concert with antimicrobial peptides hence the cellular and humoral division is arbitrary.

1.2.5.1 Insect haemocytes

The insect haemocoel is an open circulation system consisting of sessile and freely circulating haemocytes in the haemolymph, which maintains the insect hydrostatic skeleton (Lavine and Strand, 2002). The haemocytes function in transport of nutrients, distribution of hormones, removal of waste products and mediation of immune responses (Lavine and Strand, 2002). Many classification systems have been proposed based on microscopic morphology, observed functions and *in vitro* behaviour (Gupta, 1991; Ratcliffe and Walters, 1983; Price and Ratcliffe, 1974). Ratcliffe (1993) reviews the six generally recognised classes of haemocytes: namely prohaemocytes, plasmatocytes, granular cells (granulocytes), cystocytes, spherule cells (spherulocytes) and oenocytoids. Five cell types are generally observed in Lepidopterans: granulocytes, plasmatocytes, spherulocytes, prohaemocytes and oenocytoids in order of decreasing abundance (Lavine and Strand, 2002; Mulnix and Dunn, 1995). Classification systems based on surface protein components have recently been developed by discriminating haemocytes using lectins (McKenzie and

Preston, 1992; Richards and Ratcliffe, 1990; Nappi and Silvers, 1984; Rizki and Rizki, 1983) and monoclonal antibodies (Trenczek, 1998; Willott *et al.*, 1994; Mullett *et al.*, 1993). Experimental findings and general observations suggest granulocytes and plasmatocytes to have roles in anti-microbial immunity, and are hence named immunocytes (Gupta 1991). However, the phagocytic competence and frequency of immunocytes amongst the total haemocyte population vary widely between insect species and also the pathogenic microorganism to which insects are exposed (Trenczek, 1998; Ratcliffe, 1993).

1.2.5.2 Phagocytosis

Phagocytosis is an important cellular process ubiquitous to vertebrate and invertebrates to remove foreign or unwanted particles from the host, including invaders such as fungi and bacteria (Wilson and Ratcliffe, 2000; Vilcinskas and Gotz, 1999). Work is in progress to identify the molecular components in insect cell phagocytosis using *Drosophila* haemocyte cell-line mutants (Pearson *et al.*, 2003). The molecular mechanism of phagocytosis has been characterised in depth in mammalian phagocytes, involving intricate processes in recognition with multiple receptors, particle internalization, microbial killing activation, induction of inflammatory cytokines and chemokine synthesis (reviewed by Underhill & Ozinski, 2002). Phagocytosis requires active membrane dynamics driven by the host cell actin cytoskeleton (Castellano *et al.*, 2001). Recognition of non-self is facilitated by cell surface receptors; specific antibody binding Fc receptors, complement binding complement receptors and opsinin-independent mannose receptors and the actin rearrangement requires signalling cascades regulate the Rho family GTPases which act as molecular switches to reorganise of the actin cytoskeleton structure (Etienne-Manneville and Hall, 2002).

1.2.5.2.1 Fc receptors (FcR)-mediated phagocytosis

FcR-mediated phagocytosis is an antigen-specific response in vertebrate immune response, which is absent in insect immunity. Upon receptor (FcR) and ligand (anti-body) binding, the activated FcRs recruit receptors to the contact site to initiate signalling pathways leading to inflammatory mediator release, gene expression and

actin cytoskeleton rearrangement to extend pseudopodia towards the antibody bound bacterium (Garcia-Garcia and Rosales, 2002; Cox and Greenberg, 2001; Daeron, 1997). Once the protrusion spreads around the labelled object, fusion of the membrane forms a phagosome completely engulfing the invader for subsequent elimination (Tjelle *et al.*, 2000).

1.2.5.2.2 Complement receptors (CR)-mediated phagocytosis

In contrast to antibody coated particles, those opsonised with complement fragments sink into the cytoplasm of the phagocyte in the absence of extension of pseudopodia (Castellano *et al.*, 2001). The complement receptor type 3 (CR3) is $\alpha_M\beta_2$ integrin, which binds to its ligand (C3bi) on the surface of the complement-opsonised particle. CR3-C3bi interaction leads to the activation of signalling pathways that in turn recruit cytoskeletal proteins required for the formation of stress fibres and focal adhesions and also contractility for phagocytosis.

1.2.5.2.3 Mannose receptor

The mannose receptor is an opsinin-independent pattern recognition receptor capable of mediating internalisation of soluble or particulate ligands (Engering *et al.*, 1997). MRs are widely distributed and present on macrophage and dendritic cell surfaces implying immune functions (Taylor and Drickamer, 1992). MRs have eight carbohydrate recognition domains (CRDs) with affinity for carbohydrates at the termini of pathogens which are rarely found on mammalian glycoproteins providing a simple method of differentiating between self and non-self (Weis *et al.*, 1998). Other than enhancing phagocytosis, MRs also lead to complex array of downstream events (reviewed by Fraser *et al.*, 1998), including the up-regulation of macrophage FcR phagocytosis (Murai *et al.*, 1996; Murai *et al.*, 1995).

1.2.5.2.4 Molecular components of phagocytosis

The use of specific enzyme inhibitors, molecular tools to express functional or dominant negative signalling components, various functional assays and ever

advancing immunological and imagery techniques are beginning to disentangle the complex signalling pathways involved in the rearrangement of actin cytoskeleton (Castellano *et al.*, 2001). Recent research has also revealed three important members of the Rho GTPase family involved in the control and organisation of the actin cytoskeleton (Hall *et al.*, 2000; Chimini *et al.*, 2000). Yeast has been used as a main tool to dissect the immense complexity biochemical details and functions of GTPases, presumably universal across all eukaryotes (Hall and Nobes, 2000). Many bacterial microbes have evolved strategies to modulate host actin cytoskeleton to prevent the internalisation and subsequent killing (Barbieri *et al.*, 2002; Frischknecht and Way, 2001).

Both Rac and Cdc42 of the Rho family GTPases are required for FcR phagocytosis (Massol *et al.*, 1998), while Rho is shown to be involved in CR-mediated phagocytosis (Caron and Hall, 1998). Once activated, each GTPase recruit specific sets of signalling molecules and cause respective downstream actin remodelling (Cory and Ridley, 2002). These complex cross-linking pathways are also present in insect cells including haemocytes and hence are likely potential targets of bacterial exotoxins. Understanding the roles of these molecules will aid identification of bacterial toxin mechanisms.

1.2.5.3 Nodulation

Nodulation is a multicellular process involving the aggregation of haemocytes, which occurs when too many bacteria are present for clearance by phagocytosis only. A bacterial dose greater than the threshold of 1000 per microlitre was shown to be to initiate nodule formation in *G. mellonella* (Ratcliffe and Walters, 1983). A sequence of events was proposed to occur prior to nodule formation in insects; Rowley and Ratcliffe (1993) proposed invading pathogens induce granulocyte degranulation, the granulocyte contents then attract plasmatocytes to form the nodule capsule to trap the pathogens. Rizki & Rizki (1984) showed lamellocytes to be the only cell-type involved in nodulation in *D. melanogaster*, which lacks granulocytes. Recently, Pech and Strand (1996) showed granulocytes to complete the nodule capsule in *Pseudoplusia includens* by using cell-type specific antibodies. The most recent model proposed (Dean, 2002) involves a newly described cell-type called

hyperphagocytes and very large blood cells in *Manduca sexta*. The bacteria-filled hyperphagocytes (HPs) were proposed to form the centre of nodules carrying a large number of phagocytosed bacteria, these come together to form microaggregates which are surrounded by granulocytes and further encapsulated by 'very large blood cells' to form the outermost later of the nodule. It is difficult to compare these proposed models due to the use of different bacterial pathogens and insect hosts. The use of anti-haemocyte specific antibodies that cross-react with haemocytes from diverse species could aid the identification of cell surface proteins that are common haemocytes of similar functions (Trenczek, 1998; Willott *et al.*, 1995; Willott *et al.*, 1994).

1.2.5.4 Encapsulation

Encapsulation, like nodulation, is a multicellular process, where haemocytes form a cellular barrier around and contain large foreign objects such as parasitoids. Nodule formation and encapsulation is often accompanied by melanisation, melanin is suggested to stabilise the nodules and to exert anti-microbial effects (Nappi and Vass, 1993). As previously mentioned, little is known about insect haemocytes at the molecular level, the haemocytes must distinguish between self and non-self objects in the haemocoel. Encapsulation and nodulation both involve complex interactions of non-self attachment as well as self-self adhesion, the molecular mechanisms are being elucidated in *Drosophila* (Ohashi and DeFranco, 2002). The fluctuations in the total- and differential haemocyte count in infected insect haemolymph emphasise the importance of cellular responses against infection (Gillespie *et al.*, 2000; Lanzrein *et al.*, 1998; Ghally *et al.*, 1989; Horohov and Dunn, 1983; Horohov and Dunn, 1982).

1.2.6 *M. Sexta* cellular responses

M. sexta appear to execute a multi-phasic immune response against bacterial infections (Dunn and Drake, 1983; Horohov and Dunn, 1982). The immediate phase involved an increase in haemocyte count accompanied by a major reduction of viable bacteria in the haemolymph. The viable bacteria count subsequently falls steadily as the haemocyte count recovers to resting level, the relative proportions of haemocytes

are shown to vary throughout infections (Geng and Dunn, 1989; Horohov and Dunn, 1982). Lastly, the viable bacterial count diminishes as anti-microbial peptides are synthesised and take effect (Ratcliffe, 1993). To date, haematopoiesis in most insects including *M. sexta* remains poorly understood with evidence supporting various scenarios of haemocytes lineages (Lavine and Strand, 2002).

Several proteins in *M. sexta* have been shown to influence haemocyte behaviour, providing clues to haemocyte regulation. The haemocyte aggregation inhibitor protein (HAIP) of *M. sexta* inhibits haemocytes spreading over abiotic surfaces *in vitro* (Kanost *et al.*, 1994) and the inhibition of nodulation by disruption of eicosanoid biosynthesis revealed the importance of these polyunsatuated fatty-acids also thought to mediate cellular events in host-parasite interactions in mammals (Miller *et al.*, 1994). The morphology of *M. sexta* plasmatocytes is shown to depend on the presence of metal ions, which may be of importance to metallo-enzymes present on the haemocyte surface or in the haemolymph (Willott *et al.*, 2002; Meyer-Fernandes *et al.*, 2000). These reports show that cellular immune responses to be regulated by a number of modulating factors and cooperate with various humoral pathways.

1.3 Bacterial pathogenicity

The normal bacterial flora often has beneficial or even essential roles within the host (MacDonald and Pettersson, 2000). Pathogens however are able to inflict damage or harm to the host, although the eventual outcome of these dynamic relationships depends both on the relative virulence of the parasite and the susceptibility of the host (Rath, 2003). Pathogenicity is the ability of a parasite to cause damage to its host while virulence describes the degree of pathogenicity (Wassenaar and Gaastra, 2001). As prokaryotes dominate life on earth in terms of numbers of individuals and there are more species of insects than any other groups of animals; hence it is not surprising there are numerous examples of bacteria-insect interactions. Reliable pathogen-host models are useful tools for the study of these interactions (Silva *et al.*, 2002; Tzou *et al.*, 2002).

1.3.1 Anti-haemocyte mechanisms in bacterial pathogenicity

Insect immunity is arbitrarily divided into humoral and cellular responses. Haemocytes phagocytosis is a main innate immune response against bacterial infections, which is rapid and independent of previous exposure to a pathogen (Lavine and Strand, 2002; Underhill and Ozinsky, 2002). Successful pathogens must therefore by able to escape cellular phagocytosis or survive and replicate within the haemocyte intracellular conditions. Generally, virulence depends on the pathogens invasiveness (ability to persist and grow in host) and toxigenicity (production of virulence factors including all toxins which contribute towards pathogenicity) (Wassenaar and Gaastra, 2001). In the context of this thesis, I shall describe in turn mechanisms in colonisation and common groups of host cell-targeting toxins with particular attention to Gram-negative bacterial anti-haemocyte toxins.

1.3.2 Bacterial colonisation & invasion

Pathogens colonise specific niches within the host either for favourable growth conditions or to avoid host immune defence in order to establish infection (Finlay and Falkow, 1997). While some pathogens use toxins to actively prevent phagocytosis by haemocytes (Ernst, 2000), some remain attached to host cells (Celli *et al.*, 2000) and others favour intracellular habitat (Finlay and Falkow, 1997). Pathogens must adhere to mucosal surfaces of target cells or extracellular matrix to persist and/or invade (Niemann *et al.*, 2004). Adhesion can result from non-specific interactions such as hydrophobic or electrostatic where interaction is reversible (Harshey, 2003) or permanent adhesion achieved via specific target cell surface receptors such as carbohydrates or peptide residues (Niemann *et al.*, 2004; Karlsson, 2001). Adhesins in Gram-negative bacteria are divided into two groups: pili (fimbriae) and non-pilus (afimbriae) (Hultgren *et al.*, 1993). Pathogens are reported to each carry an array of adhesins and their interactions with host targets govern disease process, host susceptibility and reflect directly in symptoms during infection (Mouricout, 1997).

1.3.3 Bacterial toxins

Pathogenic bacteria produce toxins (endotoxins and exotoxins) that are directly or indirectly toxic to host cells, collectively termed virulence factors (Wassenaar and Gaastra, 2001). Endotoxins are non-protein bacterial components that are toxic to host cells via non-enzymatic mechanisms such as LPS (Alexander and Rietschel, 2001). Exotoxins are secreted or surface-associated microbial proteins, usually enzymes, that kill or modulate host cell functions at relatively low concentration (Finlay and Falkow, 1997). Exotoxins require accessory proteins to be secreted out of the bacteria (Thanassi and Hultgren, 2000a).

1.3.3.1 Lipopolysaccharides

Lipopolysaccharides (LPS) are complex amphiphilic molecules in the outer membrane surface of Gram-negative bacteria (Heumann and Roger, 2002). LPS is released from multiplying and disintegrating bacteria, the toxic component of LPS is the lipid moiety Lipid A which activates Toll-like receptors on the surfaces of macrophages causing pathophysiological response that are potentially lethal (Darveau, 1998). Endotoxins differ from exotoxins in their chemical nature at 10 kDa and heat-stable, LPSs are also relatively less potent but can cause wide ranging physiological distress leading to death (Finlay and Falkow, 1997; Lubran, 1988). In additional, the long hydrophilic polysaccharide chains of LPS, although not toxic are able to prevent complement deposition increasing the pathogen's chance of survival (Moran *et al.*, 1996).

1.3.3.2 Apoptosis inducers

Apoptosis or programmed cell death is required for the development and homeostasis of multicellular organisms and must be under stringent regulation to avoid deleterious tissue damage (Fadeel, 2003). Many complex pathways balance anti- and pro-apoptotic signals, many pathogens have evolved to exploit these signals and cause death of immune competent host cells (Navarre and Zychlinsky, 2000).

The effectors of apoptosis are caspases, which form a family of cysteine proteases that cleave after an aspartic acid (Alnemri, 1997). Caspases can initiate apoptosis by removing the inhibitor from a caspase-activated deoxyribonuclease (CAD) and inhibitor complex to allow DNA fragmentation (Enari *et al.*, 1998). Caspases can also dissociate regulatory domains from pro-apoptotic proteins effectors (Cryns and Yuan, 1998) or they can cleave nucleus structural proteins or cytoskeletal proteins to promote apoptosis (Kothakota *et al.*, 1997). The Bcl-2 family have 15 members of both pro- and anti-apoptotic proteins that can heterodimerise and determine cell fate by regulating the concentration of free proteins (Budd, 2001).

Many bacteria activate host cell apoptosis as a strategy to subvert normal host immune responses (Grassme *et al.*, 2001; Navarre and Zychlinsky, 2000). Many bacterial toxins work to disrupt the integrity of host cells to cause apoptosis indirectly, these include pore-formers, protein synthesis inhibitors, actin modulating toxins and type III secreted proteins (Finlay and Falkow, 1997) some of which are described below.

1.3.3.3 Cytolysins

Cytolysins lyse target cells with variable host- and cell-type specificity; some cytolysins have evolved to target specific host cell-types while others lyse host cells non-specifically to cause host damage (Welch, 2001). Many characterised cytolysins display potency against haemocytes, presumably to suppress the host cellular immune response (ffrench-Constant *et al.*, 2003). Additionally, lysed haemocytes provide a source of iron essential for bacterial growth in an otherwise iron-deficient microenvironment, ultimately enhancing pathogen survival (ffrench-Constant *et al.*, 2003; Martinez *et al.*, 1990).

Haemolysins are divided into three groups by their mechanism of cell lysis, which can be distinguished by studying the reaction kinetics; enzymatic, pore forming and surfactants (Rowe and Welch, 1994). Cytolysins cause lysis by enzymatic disruption of target cell membrane do so with high substrate specificity and stringent reaction conditions (Rowe and Welch, 1994). *Clostridium perfringens* α-toxin is an example

of a chromosome-encoded enzymatic cytolysin possessing phospholipase C sphingomyelinase activity (Clark *et al.*, 2003). *C. perfringens* α-toxin promotes membrane disorganisation by zinc- and calcium-dependent hydrolysis of phospholipids shown to be active against mammalian blood cells (Ochi *et al.*, 2003). Cytolytic pore-forming toxins function by insertion into host cell membrane leading to host cell lysis (Rowe and Welch, 1994). The RTX (repeats in toxin) family consists of pore-forming toxins from a wide range of bacterial pathogens covering many target hosts (reviewed by Welch, 2001). These toxins share a common genetic organisation and are secreted through the Type I secretion system. Their regulation of expression, active structure and association with lipopolysaccharide (LPS) are being elucidated (Welch, 2001). Surfactant cytolysins are peptides with hydrophobic regions rendering them amphipathic and hence surface active. They possess detergent-like action, causing lysis by solubilising target cell membrane (Rowe and Welch, 1994). An example is the *E. coli* enterohaemolysin (Jurgens *et al.*, 2002).

1.3.3.4 Actin modulators

Bacterial pathogens utilise several strategies to modulate host cell actin cytoskeleton organisation (reviewed by Barbieri *et al*, 2002). Modulation of the actin cytoskeleton allows the bacterial to resist phagocytosis or to invade specialised cells. Bacterial toxins can target either actin (the physical building blocks of the cytoskeleton) or monomeric GTP-binding proteins (the regulating molecular switches in actin organisation) (Hall and Nobes, 2000; Lerm *et al.*, 2000). Toxins can act directly on the GTPases by stable covalent modification or indirectly via other means to alter the GTPase nucleotide state causing transient modulation (Barbieri *et al.*, 2002). With so many bacterial toxins targeting the host cell actin cytoskeleton, the actin phenotype of host cells provide good indication of presence of active toxins, in the case of the present study, *Photorhabdus* toxins against *M. sexta* haemocytes.

Actin-targeting ADP-ribosyltransterases (ARTs) catalyse the ADP-ribosylation of globular actin, preventing the formation of filamentous actin and dissolve the host cell cytoskeletal structure. Examples of bacterial toxins include *C. botulinum* C2

(Wex *et al.*, 1997) and *Salmonella* spp SpvB (Lesnick *et al.*, 2001). Other toxins such as *Listeria monocytogenes* ActA (Welch *et al.*, 1998) and *Shigella flexneri* Ipa (Sansonetti, 2001) modify the cytoskeleton by altering the actin nucleation complex important for actin bundling.

Rho GTPases-targeting toxins catalyse covalent modifications via various enzymatic reactions: ADP-ribosylation (*Clostridium* C3 exoenzymes, Wilde and Aktories, 2001), glucosylation (*C. difficile* Toxin A and Toxin B, Just *et al.*, 1996, Selzer *et al.*, 1996) and deamidation (*E. coli* CNF, Lerm *et al.*, 1999; and *Bordetella* spp. DNT, Schmidt *et al.*, 1999). The altered Rho GTPase can cause different actin structural changes dependent on the molecular interactions disrupted are a result of toxin modification (Barbieri *et al.*, 2002). Some ADP-ribosyltransferase toxins target heterotrimeric G proteins, which relay signals from the cell membrane via stimulatory (G_s) or inhibitory (G_i) G-proteins. Toxin examples include cholera toxin (Vaughan and Moss, 1997) and Pertussis toxin (Locht and Antoine, 1995).

The activity of the GTPases is dependent on their nucleotide state, GTP-bound GTPases are active and are subsequently hydrolysed to GDP upon enzymatic activity, GDP-bound GTPases are inactive (Hall and Nobes, 2000). Guanine nucleotide exchange factors (GEFs) recycle the GDP-bound GTPases for GTP while GTPase-activating protein (GAPs) facilitates GTP-bound GTPases activity (Hall and Nobes, 2000). Toxins targeting GAPs and GEFs are delivered into target cells via the type III secretion system and can exert transient effects that is reversible (Barbieri *et al.*, 2002). Examples include *Salmonella* SopE (Hardt *et al.*, 1998), *Yersinia* spp. YopE (Von Pawel-Rammingen *et al.*, 2000), *P. aeruginosa* ExoS and ExoT (Henriksson *et al.*, 2002).

B. anthracis Edema factor and *P. aeruginosa* ExoY are adenylate cyclases that alter GTPases by increasing cellular cAMP level to modulate the actin cytoskeleton via cAMP-dependent kinases and phosphatases either through the A kinase-anchoring proteins (Diviani and Scott, 2001) or GEF (de Rooij *et al.*, 1998). Other toxins targeting the actin cytoskeleton have mechanisms yet to be elucidated, one example is the *Vibrio cholera* RTX which cross-links actin monomers in the host cell (Fullner and Mekalanos, 2000).

1.3.3.5 Bacterial secretion systems

Toxins rely on accessory proteins for their secretion into extracellular medium or surface-bound or delivery directly into target cells. There are several common secretion mechanisms varying in the number of components and complexity (reviewed by Thanassi and Hultgren, 2000a). Enormous sequence and experimental data gathered to date revealed the importance of secretary pathways to the understanding of bacterial pathogenesis.

All bacteria depend on the Sec system to translocate proteins across the cytoplasmic and inner membrane (IM), while gram-negative bacteria faces the challenges to cross the periplasm and outer membrane (OM). Six overarching pathways are described in gram-negative bacteria and four of these depend the Sec system, which is essential for cell viability and is present in the Archaea and Eukarya (Pohlschroder *et al.*, 1997).

The Sec-dependent secretion pathways include the autotransporters with periplasmic intermediates able to insert its C-terminus to form a β-barrel secretion channel through the OM to release protein into extracellular medium by proteolysis, a prototypical member is *Neisseria gonorrhoeae* IgA1 protease amongst others with putative role in bacterial pathogenicity (Henderson *et al.*, 1998). The similar 'single accessory pathway' requires a separate protein to form the β-barrel channel such as the *Serratia marcescens* haemolysin ShlB (Schiebel *et al.*, 1989). The lack of sequence homology between the single accessory proteins and the autotransporters implies the pathways to have evolved independently employing diverse mechanisms (Thanassi and Hultgren, 2000a).

The 'chaperone and usher' pathway also required the Sec system, the chaperone recognises periplasmic protein targets by a C-terminal signal and transports them to the OM bound usher which form oligomeric channel structures for protein secretion (Thanassi *et al.*, 1998). The uropathogenic *E. coli* P- and type I pili are organelles assembled by this pathway (Thanassi and Hultgren, 2000b). Type II secretion pathway is also Sec-dependent and related to type IV pili which is important for the virulence of many pathogenic bacteria (Russel, 1998). Pugsley *et al.* (1997) estimate

between 12 and 16 accessory proteins to make up the secreton, with most components IM associated providing energy required for secretion through the OM channel. *Klebsiella oxytoca* pullulanase represents the prototypical type II pathway (Thanassi and Hultgren, 2000a).

Three other pathways are Sec-independent and target proteins are secreted directly from the cytoplasm across the OM without any periplasmic intermediates; Type I, Type III and Type IV. Type I or ATP-binding cassette (ABC) protein exporters are present in gram-negative bacteria for secretions of numerous toxins, protease and lipases (Binet *et al.*, 1997). The Type I substrates possess a C-terminal signal of ~60 amino acids (Binet *et al.*, 1997) and interacts with the secretion apparatus consisting of an IM ABC exporter, a periplasm spanning membrane fusion protein and an OM protein.

The Type III secretion (TTSS) involves ~20 components mostly IM associated and closely related to flagellar basal body (Hueck, 1998). The *Yersinia* spp. TTSS exports effectors directly into host cell cytosol to disrupt signalling pathways and manipulate host cell behaviour in order to evade host immune responses (Cornelis, 2002). The Type IV system is closely related to the plasmid conjugation systems and the T-DNA translocation of *Agrobacterium tumefaciens* containing 11 genes within the *virB* loci (O'Callaghan *et al.*, 1999). This has been identified in *Legionella pneumophila* (Segal *et al.*, 1999; Vogel and Isberg, 1999), *Helicobacter pylori* (Covacci *et al.*, 1999) and others.

1.3.4 *Photorhabdus* and insect immunity interactions

It has been postulated that *Xenorhabdus* and *Photorhabdus* pursue an intracellular life in the insect host (Owuama and Saunders, 2003; Forst *et al.*, 1997). This hypothesis is supported by data from the *P. luminescens* W14 sample sequence analysis which revealed homologues of *Yersinia ail* and *E. coli invasin* (ffrench-Constant *et al.*, 2000). Additionally, transmission electron microscopic images of *P. luminescens* W14 particulate preparations and that of *E. coli* expressing W14 TcdAB and TccC reveal 'lollipop' structures comparing closely to the *Yersinia* YadA trimer (Hoiczyk *et al.*, 2000). Moreover, some Tc domains carry homology to *Salmonella*

SpvA and SpvB, which enhances the survival of the bacteria in macrophages (Libby *et al.*, 2000). While C-terminal region of SpvB has been shown to be an ADP-ribosyltransferase, the Tc homologous N-terminal has unknown function, Waterfield *et al.* (2001) suggests Tcs are likely to be fusion proteins of several different moieties of differing functions.

Silva *et al.* (2000) examined the growth of *P. luminescens* W14 in various insect tissues in an infected *M. sexta*. Preferential growth of *P. luminescens* W14 was found in the insect midgut and haemolymph relative to the fat body and the rest of the insect carcass. GFP-expressing *P. luminescens* W14 was observed to colonise the anterior midgut, which proceeds to the posterior midgut as infection develops. Scanning electron micrographs indicate that W14 bacteria reside underneath the extracellular matrix surrounding the midgut. However, re-examination of the transmission electron micrograph indicates the possible presence of phagocytes full of bacteria adhered to the midgut. Furthermore, it is not yet known if midgut colonisation is a unique behaviour of *P. luminescens* W14.

Adherence to specific tissue to avoid the host immune system surveillance can be described as a passive way to circumvent clearance from the larval haemolymph (Niemann *et al.*, 2004; Finlay and Falkow, 1997); more active strategies are exercised once the pathogen is exposed by host immunity (Finlay and Falkow, 1997). Some bacteria prevent immune activation by degrading infection-induced signalling molecules, while others aim for effecter molecules such as degrading antibacterial peptides or prevent the assembly of compliment components to avoid opsinisation for phagocytosis (Ernst, 2000).

An extracellular proteinase secreted by *H. bacteriophora-P. luminescens* complex or *P. luminescens* alone was found to degrade a host cecropin-like molecule in *G. mellonella* (Jarosz, 1998). Interestingly, Yamauchi (2001) isolated two novel antibacterial defensins from *Anomala cuprea in vitro* active against *Xenorhabdus japonica*, which is a pathogen of the larvae. This implies the pathogen can either prevent the production of these immune proteins *in vivo* or is able to neutralise them to enable successful pathogenesis. Intriguingly, the *Yersinae* life style demands it to provoke uptake into non-phagocytic cells as well as inhibiting uptake into immune

competent macrophages. The *Yersinia* type III secretion system (TTSS) secrets proteins collectively known as Yops which mediates effects on target host cells, ultimately neutralising host cell phagocytosis (Grosdent *et al.*, 2002; Forsberg *et al.*, 1994). *P. luminescens* W14 were observed to adhere haemocytes (ffrench-Constant *et al.*, 2001) and *P. luminescens* W14 sample sequence analysis revealed the presence of various Yops homologues (ffrench-Constant *et al.*, 2000). In addition there are homologues to ADP-ribosyltransferases potentially acting to disrupt actin cytoskeleton preventing target cell function.

1.3.4.1 *Photorhabdus* anti- haemocyte strategies

Recent efforts to identify virulence factors from bacteria or bacteria-nematode complex cell-free supernatant combine host mortality counts with *in vitro* haemocyte monolayer assays. *Locusta migratoria* fed on *Heterorhabditis megidis* carrying *P. luminescens* die 24 hours after oral consumption, however, haemocytes extracted at 12 h post injection showed impaired ability in phagocytosis (van Sambeek and Wiesner, 1999). The authors also demonstrate a reduction of phagocytic capacity in haemocytes treated with bacterial supernatant *in vivo*. Similar observations were reported in *P. luminescens* W14 supernatant against *M. sexta* haemocytes (ffrench-Constant *et al.*, 2003; Dean, 2002; Silva *et al.*, 2002).

Brillard and colleagues (2001) performed a screen of various *Xenorhabdus* and *Photorhabdus* species for cytolytic activities against *Spodoptera littoralis* haemocytes as well as sheep and rabbit red blood cells. *X. nematophilus* F1 was distinguished and further analysis revealed two distinct cytolytic activities, the first appears when bacterial growth enters stationary phase and is active both on insect granulocytes and sheep red blood cells. The second activity occurs later in stationary phase, causing haemolysis of insect plasmatocytes and rabbit red blood cells (Givaudan and Lanois, 2000). The flhDC dependent cytolysin has been isolated and termed α-Xenorhabdolysin (αX), electrophysiological and pharmacological studies indicate αX to target cell plasma membrane, disrupt cell permeability and cause eventual cell death by colloid-osmotic lysis (Ribeiro *et al.*, 2003). The authors did not observe haemolytic activities in the *Photorhabdus* species tested.

Recently, Brillard *et al.* (2002) studied *phlBA* in *P. luminescens* subsp. *laumondii* strain TT01 based on its sequence homology to calcium-dependent pore-forming haemolysins such as *Serratia marcescens* (ShlA), *E. tarda* (EthA), *Proteus mirabilis* (HpmA), and *H. ducreyi* (HhdA) and the putative haemolysin of *Y. pestis* (YhlA). Reporter and promoter construct experiments revealed its iron concentration dependent expression suggesting a role for host cell lysis in acquisition of iron for growth (Brillard *et al.*, 2002). However, the *phlBA* knockout TTO1 mutant showed no significant difference in virulence between mutant and wildtype (Brillard *et al.*, 2002). PhlA appears not to be necessary for TTO1 pathogenicity towards the lepidopteran *Spodoptera littoralis*, it is not known if exogenous expression PhlA is sufficient for pathogenicity. Given the insects' efficient immune system, *Photorhabdus* are likely to perform several lines of attack hence certain degrees of redundancy must be expected. Therefore the lack of impact on virulence in the absence of particular gene(s) such as *phlBA* does not necessarily discount a role in *in vivo* infection.

There is evidence to suggest *Xenorhabdus* and *Photorhabdus* can suppress immune responses by disrupting immune pathways other than direct lysis of haemocytes. The eicosanoid biosynthesis pathway (Tunaz *et al.*, 2003) and the phenoloxidase (PO) activation cascade (Nappi and Vass, 1993) have been shown to be critical in mediating cellular immunity in insects. Therefore these pathways represent potential targets for bacterial pathogens.

Halwani & Dunphy (1997) isolated a LPS-binding protein-1 (LBP-1) from *G. mellonella* that binds to *X. nematophilus* LPS and subsequently blocks the inhibition of phenoloxidase activation suggesting LPS to be the inhibitor responsible. Da Sliva *et al.* (2000) also demonstrated that membrane components from live or dead *X. nematophilus* are able to inhibit phenoloxidase activation in *Acheta domestica.* Additionally, *P. luminescens* W14 has also been shown to secrete a small heat-stable factor into culture supernatants which directly inhibits phenoloxidase (ffrench-Constant *et al.*, 2003). Several other groups have provided data supporting the targeting of the eicosanoid biosynthesis pathway (Park *et al.*, 2003; Tunaz *et al.*, 2003).

Aims of the project

Photorhabdus are entomopathogens that enter insect hosts passively via their nematode symbionts. Upon release into the insect haemocoel, the bacteria must contend with the insect immune responses. Haemocytes constitute the cellular components of the insect immune system with the function of physical entrapment of intruding microorganisms via phagocytosis or nodulation. This thesis aimed to characterise the effects of *P. luminescens* W14 exotoxins on haemocytes of a Lepidopteran model host *M. sexta.* The altered haemocyte phenotypes (viability and actin structure) and impeded haemocyte function (phagocytosis) were characterised using *in vitro* haemocyte monolayer assays as well as *in vivo* infections of the model host. These phenotypes formed the basis of further investigations to assess the role of candidate virulence factors in anti-haemocyte toxicity and during insect infections. Additionally, haemocyte phenotypes against a non-pathogenic *E. coli* were also documented firstly for comparison, and secondly to contribute to elucidate the general mechanisms of insect cellular responses against bacteria.

Chapter 2

Effects of *Photorhabdus* W14 cells and supernatant on *Manduca sexta* haemocytes

Some data in this chapter was published in Au C.P.Y., Dean, P., Reynolds, S.E. and ffrench-Constant R.H. (2004) *Cellular Microbiology* **6**(1): 89-95

2.1 Introduction

Photorhabdus must overcome the insect immune system in order to establish a successful infection. This may mean either avoiding or destroying insect haemocytes, the cells responsible for cellular immune response against bacterial infection. *M. sexta* has a robust immune system that overcomes bacterial infection more effectively than other lepidopteran larvae such as *G. mellonella* (Horohov and Dunn, 1982). Despite this, *P. luminescens* W14 grows relatively uninhibited in *M. sexta* haemolymph prior to insect death, suggesting either the insect immune system cannot detect the bacteria or that there are potent bacterial virulence factors that overcome the response (Daborn *et al.*, 2001). Sample sequence analysis of *P. luminescens* W14 (ffrench-Constant *et al.*, 2000) suggests it is equipped with a plethora of toxins, some of which are presumably used to combat the immune system of the wide range of potential hosts. The chapter is designed to establish phenotypes associated with such virulence factors.

Both *Xenorhabdus* and *Photorhabdus* secrete abundant extracellular proteins during stationary phase *in vitro*, thus these soluble factors are candidates for toxins secreted into the insect haemolymph during infection (ffrench-Constant *et al.*, 2003; Forst *et al.*, 1997). The expression profile of some of these putative virulence factors in *P. luminescens* W14 was obtained *in vitro* and *in vivo* (Daborn *et al.*, 2001). Strikingly, the relative orders in which the different virulence factors are expressed are similar

in vitro and *in vivo* despite the obvious difficulties in such comparison (Daborn *et al.*, 2001).

Previous studies in our laboratory (Silva *et al.*, 2002) established *P. luminescens* W14 and *M. sexta* as an infection model to document the interactions between W14 bacteria and specific tissue types in *M. sexta*. *P. luminescens* W14 preferentially colonised host midgut and haemolymph relative to fat body and carcass during early infection. Microscopic observations of sectioned infected larvae revealed W14 bacteria to colonise the space beneath the extracellular matrix of the host midgut in an anterior to posterior progression. This was followed by the disintegration of the midgut epithelium. The toxins responsible for the effect on the midgut epithelium are not known, although the high molecular weight Toxin complexes (Tcs) have been shown to cause similar effects (Blackburn *et al.*, 1998; Bowen *et al.*, 1998) and there is evidence for expression of several *tc* loci *in vivo* (Daborn *et al.*, 2001; Bowen *et al.*, 1998). However, the mechanism of action of Tc toxins applied orally or by injection is unclear.

P. luminescens W14 was reported to inhibit its own phagocytosis by *M. sexta* haemocytes while its supernatant suppressed the ability of haemocytes to undertake phagocytosis of *E. coli* (Dean, 2002; Silva *et al.*, 2002). Anti-phagocytic factor(s) have also been shown to be present in infected larval haemolymph (Dean, 2002; Silva *et al.*, 2002). Dean (2002) showed neither live and heat-killed *P. luminescens* W14 are subject to nodulation *in vivo*, while W14 supernatant reduces nodulation of *E. coli*. Although the anti-phagocytic factors are not yet identified, there are some potential candidates based on sequence homology to known toxins (ffrench-Constant *et al.*, 2000).

Insect haemocytes provide many toxin targets as they perform important immune functions in response to bacterial infection (Lavine and Strand, 2002; Trenczek, 1998; Gillespie *et al.*, 1997; Gillespie and Khachatourians, 1992; Gupta, 1991; Ratcliffe and Gotz, 1990). Sample sequence analysis of *P. luminescens* W14 revealed some Type III effector proteins as well as components of the Type III Secretion System (TTSS) itself (Waterfield *et al.*, 2002a; Ffrench-Constant *et al.*,

2000). The TTSS is a molecular syringe enabling direct contact of the bacteria to its target host cell to deliver bacterial factors directly (Page and Parsot, 2002; Cheng and Schneewind, 2000).

The present work examines the effects cells and supernatant of *Photorhabdus* strains and *E. coli* have on *M. sexta* haemocytes *in vivo* and *in vitro*. Here I show that the *in vivo* growth of pathogenic *Photorhabdus* strains correlates to decreasing haemocyte count. Also characterised are the effects of cells and supernatant of pathogenic *Photorhabdus* strains and non-pathogenic *E. coli* on haemocyte morphology, viability, motility and phagocytic competence. I used two different *Photorhabdus* isolates, W14 and K122, which correspond to two different *Photorhabdus* species *P. luminescens* ssp. *akhurstii* and *P. temperata* ssp. *temperata*, respectively. Independent phylogenetic studies place W14 and K122 in distant branches of phylogenetic trees with ancient divides (Marokhazi *et al.*, 2003; Szallas *et al.*, 1997). Forst *et al.* (1997) suggest *Xenorhabdus* and *Photorhabdus* to employ different pathogenic strategies dependent on the pathogenesis of their nematode symbiosis partners, which was recently demonstrated by Rosa *et al.* (2002). However, strategies between different species of the same genus have not been previously compared. The two isolates are distinguishable in that W14 displays oral toxicity against *M. sexta* while *P. temperata* K122 does not (Marokhazi *et al.*, 2003; Waterfield *et al.*, 2001a), therefore I was interested if the two strains show the same or dissimilar strategies against the insect immune system. Such comparison may identify key virulence factors for specific infection strategies enabling better understanding of bacteria-host interactions.

2.2 Materials and methods

2.2.1 Insects and bacterial strains

M. sexta were reared as described elsewhere (Reynolds *et al.*, 1985), newly moulted fifth instar larvae were used for all experiments in this study. The bacterial strains used were *E. coli* XL1-Blue, *P. luminescens* subsp. *akhurstii* strain W14 (W14;

Bowen *et al.*, 1998), *P. temperata* subsp. *temperata* strain K122 (K122; (Wang and Dowds, 1993) and *E. coli*-GFP (Kanamycin resistant (Kn) used at 50 μg ml^{-1}). Bacterial cultures were inoculated with fresh plate colonies and routinely grown at 30°C with constant aeration either in Luria–Bertani (LB) broth or on LB agar, except *P. luminescens* W14 that was cultured in 2% Proteose Peptone No.3 (PP3) broth or on PP3 agar. Supernatant used was obtained from stationary phase cultures 48 h post-inoculation, cells were removed by centrifugation and the supernatant filter-sterilised through 0.2 μm syringe-driven filter. Bacterial cells used were harvested from exponential phase cultures (OD_{600} = 0.3 to 0.6), washed three times with phosphate-buffered saline (PBS) and resuspended in the appropriate media. Cell counts were estimated by optical density at 600nm and serial dilutions previously calibrated to known colony forming units (CFU).

2.2.2 Determination of total haemocyte count and colony forming units

Newly-moulted fifth instar *M. sexta* larvae were chilled on ice for 15 min, surface sterilized with 70% ethanol and injected with 10 μl Grace's Insect Medium (GIM, Sigma) containing either *E. coli* (1 x 10^5), W14 or K122 (100) directly into the haemocoel using a 100 μl Hamilton syringe with a 30-gauge needle. At fixed time points over a period of 60 h post-injection, the insects were chilled on ice for 15 min, then cut at the midpoint of the dorsal horn and bled into a pre-chilled sterile polypropylene tube. Aliquots of haemolymph (50 μl) were immediately added to pre-chilled GIM (450 μl). The total haemocyte count (THC) was determined microscopically at 100x magnification with a haemocytometer, the average of three counts per insect of six insects per time point was calculated. Serial dilutions of the haemolymph were plated onto appropriate nutrient agar and incubated at 30°C for 48 h; the average number of colony-forming units (CFU) present in the haemolymph of six insects per time point per treatment was recorded.

The THC and CFU per insect were calculated assuming 320 μl of haemolymph per insect (average haemolymph volume of newly moulted fifth instar larvae was 320 ± 84 μl, n = 10) and 50 μl per insect after death (average haemolymph volume of infected larvae post death was 48 ± 18 μl, n = 10). These values of mean and

standard deviation were obtained by bleeding 10 randomly selected first-day fifth instar larvae following the exact procedure as for experimental larvae (described in section 2.2.3). Accurate measurement of individual insect haemolymph volume was not possible due to the rapid haemolymph collection procedure necessary to minimise haemocyte damage and deterioration over time *in vitro*.

2.2.3 *In vitro* haemocyte monolayers with cells or supernatant treatments

Newly-moulted fifth instar larvae were chilled on ice for 15 min, surface sterilized with 70% ethanol and bled into pre-chilled GIM and cell density was adjusted accordingly to ~5 x 10^6 cells ml^{-1}. 100 µl aliquots of haemocyte suspension were seeded onto each glass coverslip (10 mm in diameter) and left undisturbed at room temperature for 1 h to allow haemocytes attachment. The monolayers were washed three times with GIM then placed in 360 µl of GIM in a single well of a sterile 24-well plate (Nunc) and then 40 µl (10%) of cells or supernatant treatment was added and the samples were incubated at 30°C for 4 h. The monolayers were then washed three times in GIM and fixed with paraformaldehyde (PFA; 4% in PBS) incubated at room temperature for 10 min. The monolayers were washed in PBS and placed into PBS filled wells and haemocytes visualised with a NIKON light microscope at x200 magnification. Images were captured with a NIKON coolpix-990 digital camera and visualised using Adobe Photoshop 5.

2.2.4 *In vitro* haemocyte viability assays

Haemocyte monolayers prepared as described above (section 2.2.3). All monolayers were incubated with 10% treatment (dilutions in 2% PP3) at 30°C. At designated time points over 24 h, monolayers were removed from the wells, washed three times with GIM and stained with trypan blue (0.02% in PBS) at room temperature for 10 min, washed three times with PBS and then fixed with paraformaldehyde (4% in PBS). Trypan blue stained haemocyte counts and total cell counts were recorded with a light microscope at 200x magnification.

2.2.5 Protein concentration of bacterial supernatants and SDS-PAGE

P. luminescens W14 culture was grown in 2% PP3 aerated at 30°C over 60 h, optical density readings at 600 nm were taken at regular intervals. Supernatant samples extracted from the culture were filter-sterilised and stored at 4°C until use. Supernatants were concentrated using phenol:chloroform precipitation by adding 250 µl (0.5 vol) of equilibrated phenol to 500 µl of supernatant sample and mixed using vortex. Samples were centrifuged at 13k rpm for 10 min and the aqueous phase removed leaving any white precipitates at the interface of the phenolic layer. 1250 µl (5x vol) of pure ethanol was added to the phenolic phase and left at -20°C for 16 h. The samples were then centrifuged at 13k rpm for 10 min to pellet the precipitated proteins, the supernatant discarded and pellet washed with acetone. Finally the pellet was suspended in 25 µl (0.1 original vol) of 1 x SDS loading buffer. Samples were heated at 95°C for 5 min then left to chill on ice, 25 µl were loaded into lanes of 10% SDS-PAGE gel and electrophoresis was performed at 120 mV until dye front exited the gel (~90 min). The gels were stained with Coomassie Brilliant Blue (Sigma) gently agitated at room temperature overnight. Protein bands were still visible after numerous washes with destain solution (3:1:6 of methanol:glacial acid:H_2O).

2.2.6 Phalloidin staining of haemocyte monolayers

For fluorescein-conjugated phalloidin labeling of haemocytes, paraformaldehyde fixed coverslips were incubated in a fresh solution of ammonium chloride (13.3 mg ml^{-1} in PBS) at room temperature for 10 min and washed three times in PBS. Haemocytes were then permeabilised with Triton X-100 (0.2% in PBS) at room temperature for 10 min, washed three times in PBS and inverted onto 60 µl each of FITC- (fluorescein isothiocyanate) or TRITC- (tetramethylrodamine isothiocyanate) conjugated phalloidin (Sigma) incubated in darkness at room temperature for 30 min. These were washed three times in PBS, three times in distilled water and then mounted onto slides with 5 µl of mowiol mounting reagent per coverslip. Labeled haemocytes were visualised using a Zeiss LSM-510 confocal microscope at x 60 magnification controlled by using Zeiss LSM software. The optical specification for

FITC was 488 nm emission wavelength from an argon laser, 530 nm barrier filter, blue dichroic mirror and blue excitation filter set 09. Settings for TRITC were 543 nm emission wavelength from a Helium-Neon laser and 610 nm barrier filter, green dichroic mirror and green excitation filter set 15.

2.2.7 D.I.C. microscopy on haemocyte monolayers

Haemocyte monolayers were prepared as described above (section 2.2.3). Shallow wells were created using silicon multi-well casts (Molecular Probes) with well moulds 9 mm in diameter and 1.5 mm in depth adhered to standard glass 76 x 26 mm microcopy slides. The wells were filled with approximately 27 µl of 2% PP3 or W14 supernatant and haemocyte monolayers inverted onto the wells using silicon gel to form a watertight seal. For differential interference contrast (D.I.C.) microscopy imaging, a X40 Fluor water immersion lens was used on an upright Nikon E1000 microscope. Images were collected at 3 min intervals for up to 12 h with a Hamamatsu C4880–07 camera controlled by using METAMORPH software (Universal Imaging, Media, PA).

2.2.8 *In vitro* and *in vivo* phagocytosis assays

Live GFP-expressing *E. coli* (in LB), *P. luminescens* W14 (in PP3) and *P. temperata* K122 (in LB) were grown with aeration at 30°C to exponential phase (OD_{600} = 0.3 to 0.6) and resuspended in PBS. Dead GFP-expressing *E. coli* and *P. luminescens* W14 and *P. temperata* K122 used in the phagocytosis assay were killed by incubation with paraformaldehyde (4% in PBS) at room temperature for 30 min, washed three times in PBS. Cell suspensions were streaked onto appropriate agar plates to check for complete killing of bacteria. FITC-labelling of *E. coli* and *Photorhabdus* required large volume exponential phase cultures centrifuged to pellet the bacterial cells. The pellet was incubated with paraformaldehyde (4% in PBS) at room temperature for 30 min, washed three times in PBS and resuspended in carbonate buffer (pH 9.4) with 0.1mg/ml fluorescein-isothiocyanate (FITC) (Sigma, isomer I). The suspension was incubated shaking in complete darkness at room temperature for 30 minutes. The cells were washed with PBS until the supernatant was free of

excess FITC. Bacteria were suspended at 1 x 10^8 cell ml^{-1} in PBS, passed through syringe needle of 0.5 μm to separate clumps then aliquoted and stored at -20° until use.

For the *in vitro* phagocytosis assay, haemocyte monolayers for *in vitro* assays were prepared as described in section 2.2.3. Treatment was added to each well containing a monolayer in 360 μl of GIM and incubated at 30°C for 15 min then 40 μl of either live or dead or *E. coli* -GFP or *Photorhabdus*-GFP (1x10^7 cells ml^{-1}) was added to each well and left undisturbed at 30°C for 4 h.

For the *in vivo* co-injection phagocytosis assay, fifth instar *M. sexta* larvae were prepared for injection as described in section 2.2.2. Larvae were injected with either 10 μl of PP3 or bacterial supernatant containing 1 x 10^7 *E. coli*-GFP and incubated at 25°C for 2 h. Larvae were then chilled on ice for 15 min, bled into pre-chilled GIM and monolayers prepared and haemocytes stained as described in section 2.2.6.

For the *in vivo* pre-treatment phagocytosis assay, larvae were injected with 10 μl of GIM containing 1 x 10^5 of either *E. coli*, W14 or K122; after 2 h at 25°C a second injection of 1 x 10^7 *E. coli*-GFP in 10 μl of GIM was administered and the larvae were incubated at 25°C for a further 2 h. The larvae were chilled, bled and the haemolymph was used to prepare monolayers and stained as described in section 2.2.2 and 2.2.6, respectively.

The haemocyte monolayers were visualized under the confocal microscope and the percentage of phagocytic haemocytes determined by counting TRITC-phalloidin stained haemocytes co-localised with the *E. coli*-GFP in relation to total haemocyte count. Data shown were calculated from counts of 300-600 cells per monolayer per insect and three insects per treatment.

2.2.9 Western analysis of *M. sexta* immune recognition molecules

Either haemolymph incubated with bacterial supernatant *in vitro* at 25°C for 12 h or haemolymph from *E. coli* or *P. luminescens* W14 injected larvae 12 h post-injection was mixed with 2x loading buffer at 1:1 ratio and heated at 95°C for 5 min then chilled on ice for 5 min. 20 µl of samples and pre-stained broad-range protein markers (7-175 kDa, NEB) were loaded into 10% SDS-PAGE gel, electrophoresis performed at 120 V until the loading dye-front ran out of the gel then blotted onto nitrocellulose membrane for Western analysis (Sambrook *et al.*, 1989).

The blots were used for Western analysis against several antibodies, anti-serum raised in rabbit reactive against *M. sexta* hemolin, peptidoglycan recognition protein (PGRP) and immulectin-2 (generous gifts from Dr. Kanost, KS, USA) and a commercial anti-actin (Sigma). The procedure used as described in Sambrook *et al.*, (1989) using an alkaline-phosphatase conjugated secondary anti-rabbit antibody (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma). The blots were rinsed with H_2O to stop the reaction as instructed by the manufacturer.

2.2.10 Immunofluorescent analysis of haemocytes

Haemocyte monolayers were prepared from 1) *E. coli*-GFP injected larvae either with or without W14 supernatant (*in vivo* experiments), 2) from naive larvae with the monolayers subsequently incubated with *E. coli*-GFP at 30°C for 4 h (*in vitro* experiments). Monolayers were fixed with paraformaldehyde (4% in PBS) and then incubated in a fresh solution of ammonium chloride (13.3 mg ml^{-1} in PBS) at room temperature for 10 min, followed by three washes in PBS. The haemocytes were then permeabilised with Triton X-100 (0.2% in PBS) at room temperature for 10 min with further three washes in PBS. *M. sexta* haemocyte monoclonal antibodies (generous gifts from Dr. Trenczek, Germany) were used at 1 in 100 dilution. The permeabilised haemocyte monolayers were inverted onto 60 µl of blocking solution (10% normal donkey serum in PBS) at room temperature for 1 h then transferred onto 60 µl of primary antibody in blocking solution at room temperature for 2 h.

After three 5 min washes in blocking solution, the monolayers were inverted onto 60 µl of 1:300 Cy3-conjugated anti-mouse secondary antibody (Jackson Immuno Research, USA) in blocking solution for 1 h in complete darkness. After further three 5 min washes in PBS the coverslips were mounted onto the slides by inverting the monolayer onto 30 µl Mowiol mounting reagent (Chem-Bio). An extra FITC-phalloidin staining step was required for the phenotype and cell-type specificity study, here the monolayers were inverted onto 60 µl of FITC-phalloidin in PBS (Sigma) at room temperature for 30 min in compete darkness, followed by three washes in PBS and three washes in H_2O and mount as described. Labeled haemocytes were visualized using a Zeiss LSM-510 confocal microscope at x60 magnification.

2.3 Results

2.3.1 Survival of *E. coli* and *Photorhabdus* in *M. sexta* haemolymph

To investigate how bacteria survive in *M. sexta*, non-pathogenic *E. coli* and pathogenic strains of *Photorhabdus* were injected into larval *M. sexta* and their persistence monitored. The newly moulted fifth instar larvae were injected with either 1 x 10^5 cells of *E. coli* or 100 cells of *Photorhabdus* and incubated at 25°C for up to 60 h post-injection. The larvae were bled at designated time intervals post-injection and serial dilutions plated to determine the recoverable colony forming unit and haemocytometer counts taken to estimate the total haemocyte count in the insect haemocoel.

The clearance of 1 x 10^5 cells of *E. coli* from the insect haemolymph was complete by 36 h post-injection (Fig. 2.1a). In contrast, both the *Photorhabdus* strains replicated in the larvae and reached ~1 x 10^4 cells / insect haemolymph at 24 h post-injection, ultimately reaching 1 x 10^8 cell / insect haemolymph by 60 h post-injection. Another striking difference observed is the total haemocyte count profile between the *E. coli* and *Photorhabdus* injected larvae (Fig. 2.1b). Notably, *E. coli* caused a transient increase in total circulatory haemocyte count with a peak at 8 h post-injection; the resting level of 2 x 10^6 / insect haemolymph was resumed by 12 h post-injection. By contrast, *P. luminescens* W14 caused a gradual decrease and *P. temperata* K122 caused a more immediate fall in haemocyte numbers. Both *Photorhabdus* strains caused a continual decline in total haemocyte count until insect death.

2.3.2 Effects of bacterial cells and supernatant on *M. sexta* haemocyte morphology *in vitro*

To investigate the effects of bacterial cells and supernatant on *M. sexta* haemocyte morphology *in vitro*, haemocyte monolayers were exposed to washed cells and cell-free supernatant of *E. coli* and *Photorhabdus* strains W14 and K122 at 30°C for 4 h. The monolayers were viewed under the light microscope to compare the treated

haemocyte morphology against the typical morphology of the two main immune competent haemocytes: granulocytes are normally spherical at ~10 μm in diameter and plasmatocytes adopt a spindle shape (Fig. 2.2a).

Supernatant treated haemocytes revealed that neither *E. coli* nor *P. temperata* K122 supernatant has any visible effect haemocyte morphology (Fig. 2.2c and e). However, *P. luminescens* W14 supernatant caused morphological changes to haemocytes visible under light microscopy. Granulocytes appeared flattened leaving a more prominent nucleus while plasmatocytes also appeared more spread with general increase in the cell surface area (Fig. 2.2g).

Haemocytes treated with bacterial cells showed that *Photorhabdus* cells adhered to the monolayers while *E. coli* cells were completely absent from the corresponding monolayer, presumably either phagocytosed or washed off (Fig. 2.2b, d and f). Both *P. temperata* K122 and *P. luminescens* W14 cells caused changes in haemocyte morphology changes; granulocytes became more angular in shape while the plasmatocytes flattened over the coverslip losing their normal spindle shape (Fig. 2.2d and f). The effect on plasmatocytes was also seen in *P. luminescens* W14 supernatant treated monolayers.

These data indicate anti-haemocyte factor(s) to be cell-associated in *Photorhabdus* strains K122 and W14, while W14 also possesses secreted cytotoxic factors. The observed morphological changes in the haemocytes may affect their viability, rendering them inactive in mounting an immune response.

2.3.3 Effects of bacterial supernatants on haemocyte viability *in vitro*

To determine if the W14 supernatant induced morphological changes (Fig. 2.2) lead to haemocyte death, haemocyte monolayers were exposed to *E. coli* and *Photorhabdus* supernatants to assess haemocyte viability over 24 h. The monolayers were removed and stained with trypan blue to distinguish live and dead cells at designated time points over the 24 h treatment (Fig. 2.3). Both PP3 control and *E. coli* supernatant caused less than 5% haemocyte mortality, while both supernatants

from *Photorhabdus* strain W14 and K122 dramatically reduced haemocyte viability. W14 supernatant caused an immediate reduction in viable haemocyte count down to 75.9 ± 9.7 % after 4 h, while K122 supernatant treated monolayers had 98.5 ± 0.7 % haemocytes viability at 4 h which subsequently reduced to 66.1 ± 6.9 % after 12 h of treatment (Fig. 2.3). Both *Photorhabdus* supernatants reduced haemocyte viability to less than 25% after 18 h of treatment.

The data reflect the morphological changes previously observed (Fig. 2.2) where *P. temperata* K122 supernatant treated haemocytes appeared unaffected after 4 h treatment while *P. luminescens* W14 supernatant treated displayed changes after 4 h. These observations confirm that *P. luminescens* W14 secretes cytotoxic factor(s).

2.3.4 Effects of W14 supernatant on *M. sexta* haemocyte viability *in vitro*

To examine the *in vitro* growth curve and secreted protein profile of *P. luminescens* W14, a culture was grown in 2% PP3 aerated at 30°C and its growth monitored over 60 h based on optical density at 600 nm. The *P. luminescens* W14 *in vitro* culture growth displayed the classical sigmoidal bacterial growth curve (data not shown). Cell-free supernatant extracted from samples of various OD_{600} were concentrated by phenol-chloroform precipitation and the protein contents were resolved in a 10% SDS-PAGE gel and visualised with Coomassie staining (data not shown). The protein content in the supernatant was dramatically increased in extracts from stationary phase cultures with OD_{600} reading exceeding 2. The abundance of protein in these extracts may include cytotoxic factors released from the bacteria through active secretion or by general autolysis.

To establish if the supernatant from earlier growth phases carry cytotoxic factors active against haemocytes, the cell-free supernatant samples taken from OD_{600} 0.1 to 2.2 were taken and used to treat haemocyte monolayers *in vitro* for 4 or 24 h at 30°C. The haemocytes were stained with trypan blue and the monolayers were visualised using light microscopy to determine haemocyte viability (Fig. 2.4a). The data show that all treatments after 4 h resulted low percentage cell death and most 24 h

treatments caused ~ 40% mortality except the stationary phase supernatant which caused complete haemocyte killing.

To further examine the cytotoxic factor(s) in *P. luminescens* W14 stationary phase supernatant, serial dilutions of the stationary phase supernatants were overlaid onto haemocyte monolayers for 1, 4 and 24 h followed by a further 23, 20 and 0 h incubation respectively totalling 24 h treatment. The haemocytes were trypan blue stained and the percentage viable haemocyte count recorded (Fig. 2.4b). All 24 h treatments regardless of the dilution factor caused significant cell death relative to the controls and interestingly, supernatant dilution by three orders of magnitude did not cause reduction in its cytotoxicity. This experiment revealed that haemocyte death is a slow process requiring prolonged exposure of more than 4 h to the target cells, and also that the toxin(s) responsible for haemocytes death are secreted in concentration greater than required when the bacteria are cultured *in vitro*.

2.3.5 Effects of bacterial supernatants on haemocyte actin cytoskeleton *in vitro*

To examine the effects of bacterial supernatant on the actin cytoskeleton of *M. sexta* haemocytes, monolayers were exposed to supernatant from *E. coli* or *Photorhabdus* strains K122 or W14. The haemocyte actin cytoskeleton was visualised with TRITC-conjugated phalloidin labelling. The PP3 control treated granulocytes showed a distinct polarisation of actin in the cell bodies with intense staining adjacent to the probable leading edge of the cell (Fig. 2.5a). This morphology was not affected by treatment with *E. coli* or *P. temperata* K122 supernatant (Fig. 2.5d and g). However, *P. luminescens* W14 supernatant induce the loss of actin polarisation and the granulocytes appeared shrunken with marked filopodia or microspikes (Fig. 2.5j).

The PP3 control treated plasmatocytes were spread with round or spindle-shaped cell bodies and displayed a diffuse actin cytoskeleton (Fig. 2.5b and c), which appeared unchanged after *E. coli* or *P. temperata* K122 treatment (Fig. 2.5e, f, h and i). *P. luminescens* W14 supernatant however caused dramatic changes in plasmatocyte morphology. Most commonly, plasmatocytes showed severe actin bundling (stress

fibres) throughout the cell body (Fig. 2.5k and l). Secondly, some plasmatocyte actin skeletons appeared to have been destroyed leaving only punctate pattern of staining (Fig. 2.5l). These actin dense foci may represent remnants of actin-rich points of contact between the cell and the substrate.

Haemocyte monolayers exposed to the same treatments for 24 h reveal PP3 control and *E. coli* supernatant treated granulocytes had more diffused actin distribution with less prominent polarity (Fig. 2.6a and d) than the 4 h treated cells (Fig. 2.5a and d). Both *Photorhabdus* K122 and W14 supernatant treated granulocytes appeared smaller in size with shrunken cell bodies and the W14 supernatant treated haemocytes had more prominent filopodia extension (Fig. 2.6g and j).

PP3 control or *E. coli* supernatant treatments of the spindle-shaped plasmatocytes showed intense yet diffuse actin staining and aggregation on the glass coverslip as if forming a nodule (Fig. 2.6b and e). The round plasmatocytes had less intense and diffuse actin staining (Fig 2.6c and f). The *P. temperata* K122 supernatant treatment caused these cells to spread, again losing the spindle shape with diffuse actin. Some round plasmatocytes containing punctate foci within a clearly visible cell body on the coverslip (Fig 2.6h and i) could be seen. *P. luminescens* W14 supernatant treated cells had stress fibres, punctate actin foci or peripheral actin condensation (Fig. 2.5k and l) as observed after a 4 h treatment. Note that neither of the two *Photorhabdus* supernatant treated monolayers contained micro-aggregates of haemocytes which were repeatedly seen on the PP3 and *E. coli* supernatant treated monolayers (Figure 2.6 b and e).

2.3.6 Correlation of W14 induced actin phenotypes to plasmatocyte subtypes

P. luminescens W14 supernatant treatment induced plasmatocytes to display numerous actin phenotypes such as stress-fibres, peripheral actin condensation or actin foci (Fig 2.5 and 2.6). I used specific monoclonal antibodies to test the idea that particular actin phenotypes were displayed by particular plasmatocyte subtypes, which would indicate specific targeting by anti-haemocyte factors.

Haemocyte monolayers exposed to PP3 control or *P. luminescens* W14 supernatant were fixed and labelled with anti-haemocyte antibodies with a fluorescein (Cy3) conjugated secondary antibody, followed by FITC-phalloidin staining to highlight the actin structure. The three anti-plasmatocyte antibodies used were MS#13, MS#51 and MS#77. Of the three antibodies, only MS#13 has a known molecular target; a 90 kDa plasmatocyte membrane protein shown to be important for cell spreading and encapsulation (Wiegand *et al.*, 2000), while the targets of two MS#51 and MS#77 are unknown. As granulocytes appeared uniform throughout the haemocyte population, MS#7 was used as experimental control. MS#7 stained both polarised and shrunken granulocytes after treatment with PP3 control and *P. luminescens* W14 supernatant (Fig. 2.7a and e),

Both round and spindle-shaped plasmatocytes produce actin bundles or actin foci after *P. luminescens* W14 supernatant treatment, both types of cell staining positive against MS#13 and MS#51 (Fig. 3.12b, c, f, g). It was also noted that some plasmatocytes maintained their diffuse actin cytoskeleton after *P. luminescens* W14 supernatant treatment, these cells also stained positive with MS#13 and MS#51. Unfortunately, therefore the antibodies available cannot discriminate between targeted and unaffected plasmatocytes.

Interestingly, antibody MS#77 stained a select of small granulocyte-like round cells in the PP3 control treated monolayers, but after W14 supernatant treatment MS#77 stained all granulocytes and small spread plasmatocytes and not haemocytes with induced actin foci or stress fibres (Fig. 2.7d and h).

2.3.7 Effects of *P. luminescens* W14 supernatant on haemocyte motility *in vitro*

Since *P. luminescens* W14 supernatant treatment causes loss of polarity to granulocytes and induce strong actin bundling in plasmatocytes, haemocyte motility may be affected. In order to investigate this hypothesis, haemocyte monolayers were set up and incubated with either PP3 control or W14 supernatant and cell movements were monitored with a differential interference contrast (D.I.C.) microscope and camera. Figure 2.8 displays images of the same view during incubation with the two

treatments at 0, 1 and 3 h. Movement of plasmatocytes was apparent in the monolayer exposed to PP3 control while little movement was evident in the *P. luminescens* W14 supernatant treated monolayer. Hence, W14-induced changes in morphology and in actin structure impede cell motility.

2.3.8 Effects of *P. luminescens* W14 on haemocyte phagocytic competence

Various *in vitro* and *in vivo* assays were performed to examine the interactions between live and dead *E. coli* and *Photorhabdus* strains with haemocytes. The data obtained also revealed the effects of the bacterial cells and supernatants on the ability of haemocytes to phagocytose *E. coli*. Haemocyte monolayers incubated with live or dead GFP-expressing *E. coli* or *Photorhabdus* for 4 h at 30°C were then stained with TRITC-phalloidin for visualisation. All GFP-bacteria co-localised haemocytes were scored under the confocal microscope to determine the percentage of phagocytic haemocytes (Fig. 2.9). Both *Photorhabdus* strains suppressed their own phagocytosis, while live W14 showed stronger inhibition.

To test whether the enhanced reduction in phagocytosis is owing to secreted anti-phagocytic factors present in *P. luminescens* W14 supernatant (Silva *et al.*, 2002), haemocyte monolayers were treated with cells or supernatant of *E. coli*, *Photorhabdus* K122 or W14 and their ability to phagocytose *E. coli*-GFP tested. Figure 2.10a shows that both cells and supernatant of W14 can suppress the haemocyte phagocytic competence relative to either fraction of K122 or *E. coli*. The presence of an inhibitory factor in W14 supernatant is further confirmed by the concentration-dependent inhibition shown in Figure 2.10b. Interestingly, while K122 cells are able to inhibit their own phagocytosis (Fig. 2.9), haemocytes treated with either K122 cells or supernatant remain capable to phagocytose *E. coli* (Fig. 2.10a). These observations reflect the increased haemocyte mortality (section 2.3.4) and actin phenotype rearrangement *in vitro* (section 2.3.5) with 4 h *P. luminescens* W14 supernatant treatment over the non-toxic *P. temperata* K122 supernatant treatment.

To find out if the same effect applies *in vivo*, *E. coli*-GFP were co-injected with *E. coli*, K122 or W14 supernatant directly into *M. sexta* haemocoel. The *M. sexta* larvae were bled 2 h post-injection to assess phagocytic competence of haemocytes. Figure 2.11a shows that the W14 supernatant treatment reduced the percentage of phagocytic haemocytes relative to K122 or *E. coli* supernatant, confirming that *P. luminescens* W14 secretes anti-phagocytic or cytotoxic factors which also function *in vivo*.

To determine if pre-injection of live *E. coli* or *Photorhabdus* strains would affect the haemocytes in the same manner as the supernatant, larvae were challenged with 10^5 cells of *E. coli* or *Photorhabdus* W14 or K122 prior to incubation at 25°C for 2 h. This was followed by a second injection of 10^7 cells of *E. coli*-GFP and a further 2 h incubation at 25°C, the larvae were then bled and haemocytes monolayers prepared for microscopic counts to assess the percentage of phagocytic haemocytes. Pre-treatment with injected bacteria into the insect showed no effect on the haemocytes ability to phagocytose *E. coli*-GFP (Fig. 2.11b). Although W14 supernatant from stationary phase batch cultures has inhibitory effects *in vivo*, the inhibitory effects of the bacterial cells themselves may be transient or localised *in vivo*.

To summarise, both *Photorhabdus* strains suppress their own phagocytosis by *M. sexta* haemocytes *in vitro* (Fig. 2.9) but only W14 cells and supernatant reduce the haemocytes ability to phagocytose *E. coli* (Fig. 2.10a). *P. luminescens* W14 supernatant inhibits phagocytosis in a concentration-dependent manner (Fig. 2.10b). *E. coli* co-injected with bacterial supernatant directly into the larval haemocoel revealed that W14 supernatant also suppresses phagocytosis *in vivo* (Fig. 2.11a) reflecting *in vitro* observations. Pre-treatment by infection of *E. coli*, K122 or W14 into the larval haemocoel did not result in an observable difference in the percentage of phagocytic haemocytes (Fig. 2.11b) at the time points examined.

2.3.9 Specific haemocyte subtype targeting by W14 anti-phagocytic factors

Analyses were carried out to determine if particular haemocyte sub-populations are phagocytic and whether they differ *in vivo* and *in vitro*. Haemocyte subtype specific

monoclonal antibodies were used as tools to reveal any changes in the phagocytic subpopulations after *P. luminescens* W14 supernatant treatment *in vitro* and *in vivo*. Particular attention was paid towards the recently described hyperphagocytes (HPs; see text in section 1.2.5.1).

In vitro, haemocyte monolayers were prepared from naive larvae and then exposed to *E. coli*-GFP with and without *P. luminescens* W14 supernatant treatment before staining with haemocyte subtype specific antibodies (Fig. 2.12a - h). Granulocytes treated with PP3 control phagocytosed *E. coli*-GFP (Fig. 2.12a), and were inhibited by *P. luminescens* W14 supernatant (Fig. 2.12e). Hyperphagocytes were phagocytic against *E. coli*-GFP with either PP3 control or W14 supernatant, suggesting that phagocytosis by hyperphagocytes is resistant to inhibition by W14 supernatant (Fig. 2.12b, c, f, g). Hyperphagocytes stain positive against anti-plasmatocyte antibodies but not all plasmatocytes are phagocytic. Peculiarly, MS#77 stained all haemocytes on the PP3 control treated monolayers but failed to stain any haemocytes on monolayers that had been treated with W14 supernatant (Fig. 2.12d and h).

In vivo, larvae were injected with *E. coli*-GFP suspended in either PP3 or W14 supernatant were injected directly into the haemocoel, the larvae were incubated at 25°C and haemolymph extracted for monolayer preparation 4 h post-injection. Figures 2.12i, j and k show phagocytic granulocytes and hyperphagocytes, while only hyperphagocytes were phagocytic in the presence of W14 supernatant (Fig. 2.13m, n and o) reflecting *in vitro* observations. However, these data should be interpreted with caution, as possible effects on the *E. coli* suspended in W14 supernatant, which might have altered their surface epitopes, were not taken into account.

2.3.10 Western analysis of immune recognition molecules in challenged haemolymph

To determine if certain immune recognition molecules are targeted by *P. luminescens* W14 anti-phagocytic factor(s), Western analysis of *M. sexta* hemolin, immunlectin-2 and peptidoglycan recognition protein (PGRP) was used (Fig. 2.13).

Freshly extracted whole haemolymph was incubated for 12 h at 30°C with GIM or supernatants of *E. coli* or *P. luminescens* W14 and prepared for Western analysis. Figure 2.13a shows a slight reduction in hemolin in the W14 supernatant treated sample only.

The same assessment was performed *in vivo* (Fig. 2.13b), larvae were injected with either GIM, *E. coli* or *P. luminescens* W14 cells and bled 24 h post-injection for Western analysis. The level of hemolin and PGRP appear elevated by both *E. coli* and *P. luminescens* W14 bacterial challenge relative to the GIM injected control, immunlectin-2 level appear unaffected. These data suggest the levels of recognition molecules present in the larval haemocoel are not adversely altered by *P. luminescens* W14 relative to *E. coli.*

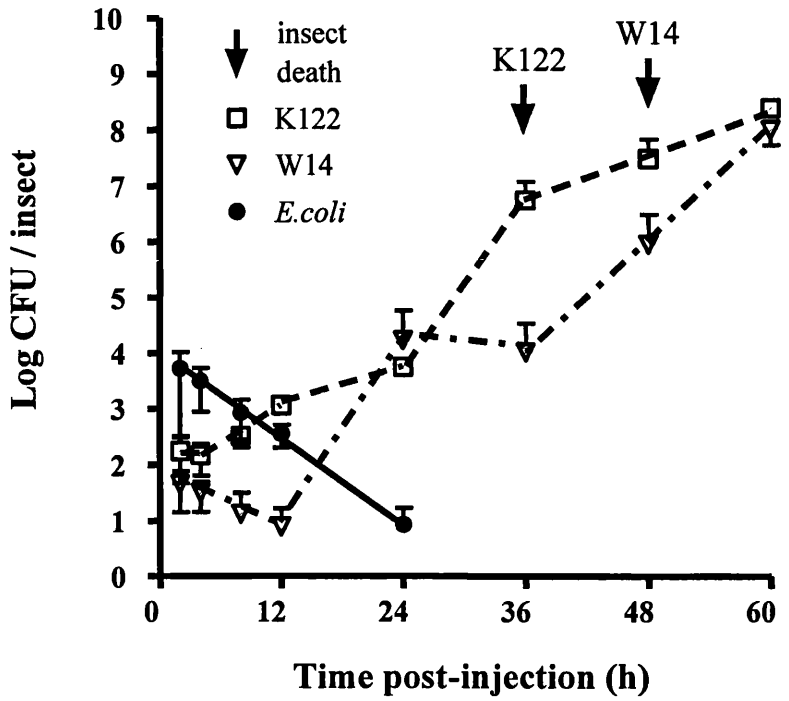


Figure 2.1a *Photorhabdus* and *E. coli* viable count in *M. sexta* haemocoel over 60 h post-injection (Au *et al.*, 2004). Larvae were injected with either 1 x 10^5 cells of *E. coli* or 100 cells of *Photorhabdus* strain W14 or K122. The larvae were bled at different time intervals to determine the number of recoverable colony forming units (CFU) in the insect haemocoel. Note that *E. coli* are cleared within 36 h post-injection, whereas both *Photorhabdus* strains grow rapidly over time. Arrows indicate the average time of insect death observed with each *Photorhabdus* strain. CFU values were calculated from plating serial dilutions of haemolymph assuming 320 µl haemolymph/insect (320 ± 86 µl, n = 10) and 50 µl per insect after death (haemolymph volume of infected larvae post death was 48 ± 18 µl, n = 10), data represent mean ± S.E. of six insects/time-point/treatment.

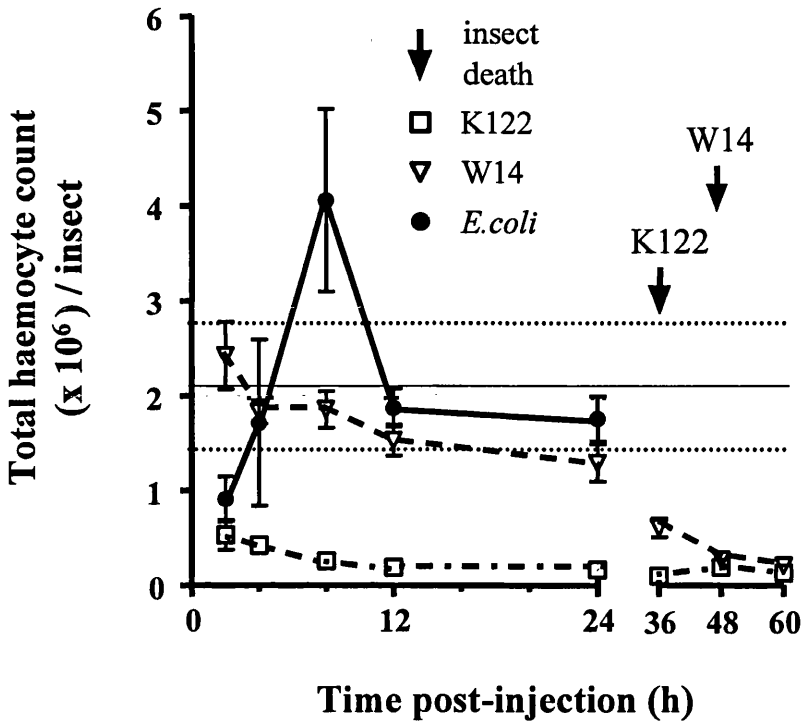
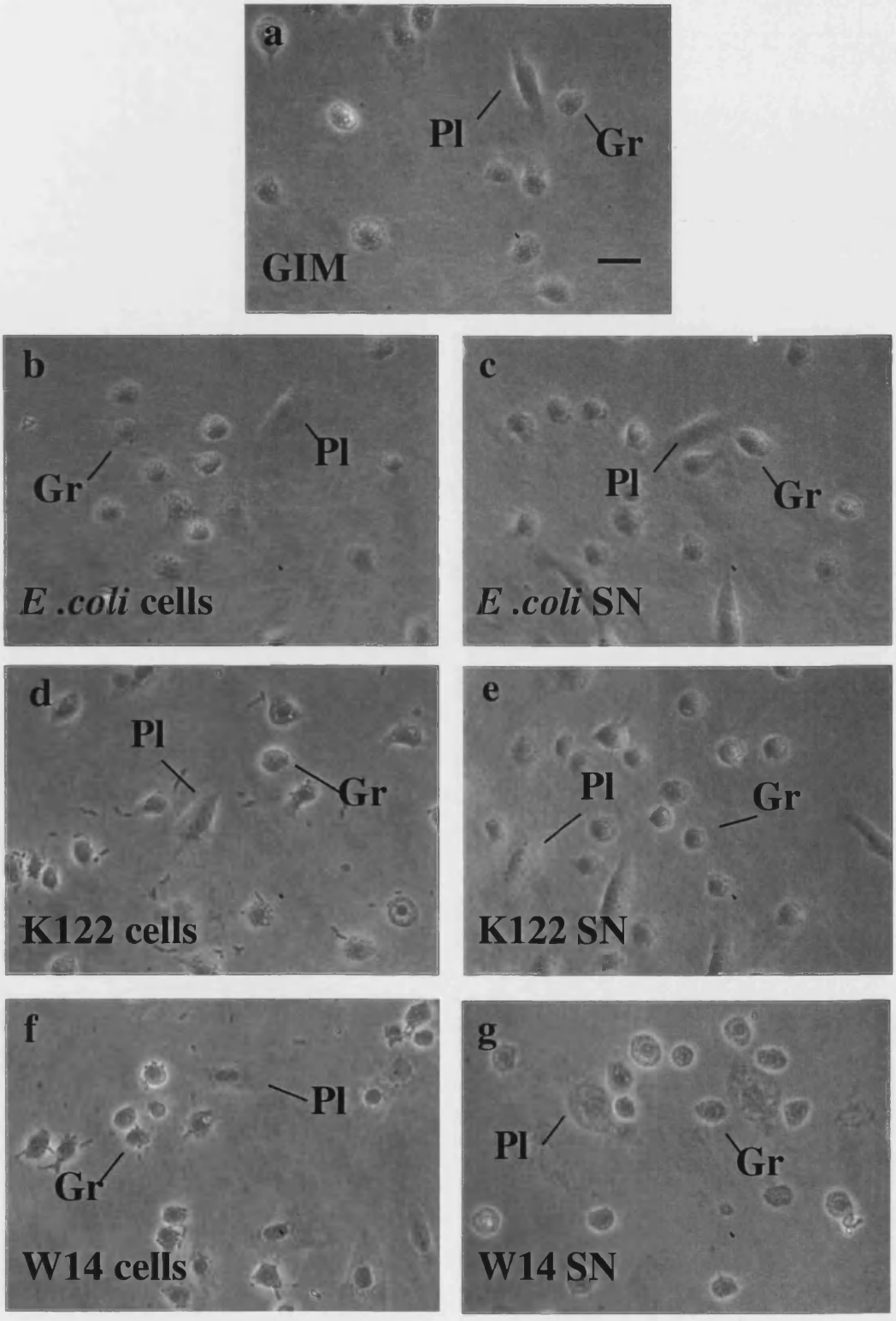


Figure 2.1b *M. sexta* total haemocyte count during *Photorhabdus* and *E. coli* infection (Au *et al.*, 2004). Larvae were injected with either 1 x 10^5 cells of *E. coli* or 100 cells of *Photorhabdus* strain W14 or K122. The larvae were bled at different time intervals to determine the total free haemocyte count (THC) over 60 h post-injection. The data show a transient increase in free haemocytes after *E. coli* injection and a decline in haemocytes following injection of either *Photorhabdus* strain. Solid line illustrates the average THC of six newly moulted fifth instar larvae with dotted lines showing standard deviation at 2.1 x $10^6 \pm 6.7 \pm 10^5$ / insect (320 µl of haemolymph). Arrows indicate the average time of insect death observed with each *Photorhabdus* strain. THC data shown represent the mean ± S.E. of three samples/insect with six insects/time-point/treatment, assuming 320 µl haemolymph/insect (320 ± 86 µl, n =10) and 50 µl per insect after death (haemolymph volume of infected larvae post death was 48 ±18 µl, n = 10).

Figure 2.2 Light micrographs of *M. sexta* haemocyte monolayers treated with cells or supernatant fractions from *E. coli* or *Photorhabdus* strain W14 or K122. Haemocyte monolayer after 4 h incubation with Grace's insect media (a) shows the typical cell morphology round granulocytes (Gr) and spindle shaped plasmatocytes (Pl). The morphology of haemocytes are compared to those treated with *E. coli* cells and supernatant (SN) (b + c), K122 cells and supernatant (d + e), W14 cells and supernatant (f + g). Note haemocytes treated with W14 cells (f) and supernatant (g) display different morphology to control treated haemocytes (a), haemocytes appear more spread against the coverslip with generally increased cell surface area. Also bacterial cells remain visible on monolayers incubated with K122 cells (d) and W14 cells (f) in contrast to the *E. coli* treated monolayer where the bacteria are phagocytosed or washed off. (Scale bar =10 μm)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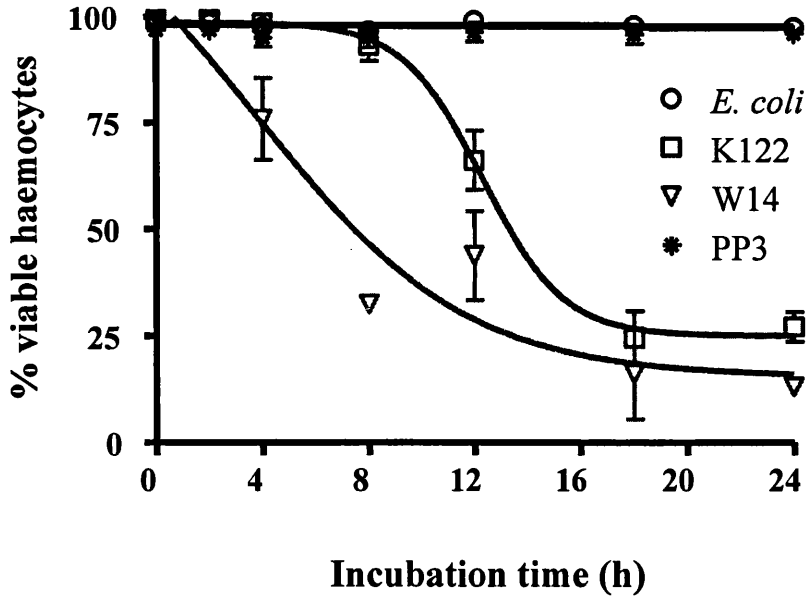

Figure 2.3 Viability of haemocytes treated with bacterial supernatants (Au *et al.*, 2004). *M. sexta* haemocyte monolayers were treated with either PP3 broth or supernatant from *E. coli*, *Photorhabdus* W14 or K122 over 24 h at 30°C. Trypan blue staining was used to discriminate between the live and dead (blue stained) cells from random views on the monolayers treated for designated time intervals. Note the decline in haemocyte viability following treatment with either *Photorhabdus* supernatant. The onset of the haemocyte death is more immediate after *P. luminescens* W14 supernatant treatment than that of *P. temperata* K122. Data represent mean and S.D. of at least 1000 haemocytes counted per monolayer per insect, three insects/time-point/treatment.

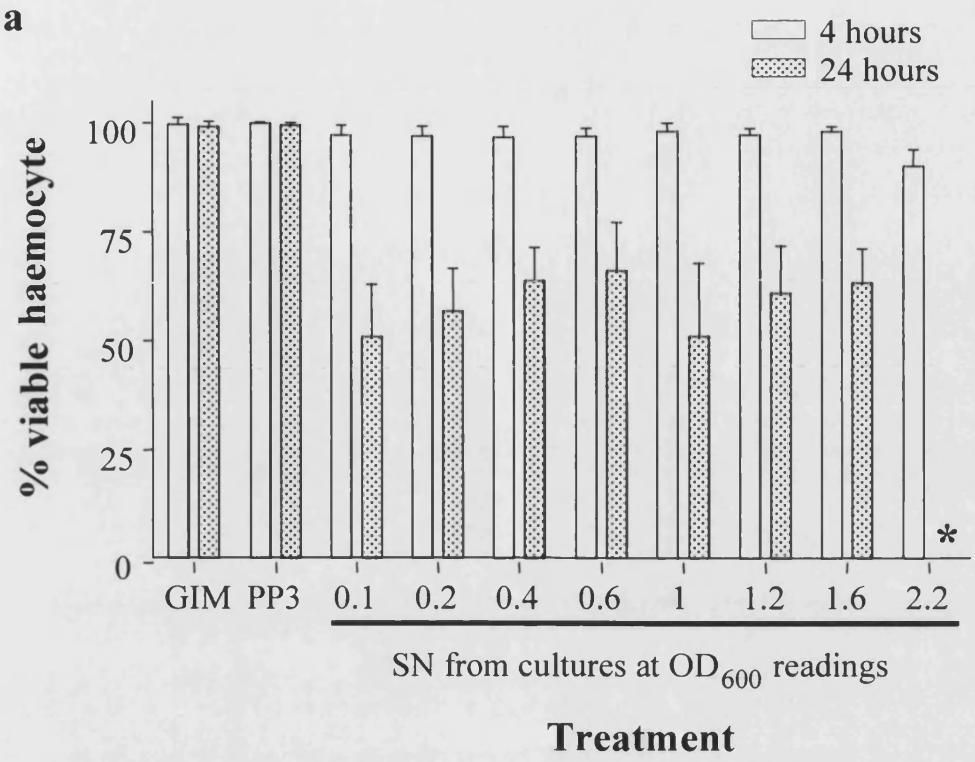

Figure 2.4a Effects of *P. luminescens* W14 supernatant on haemocyte viability. Haemocyte monolayers were exposed to 10% supernatant from various growth phases *in vitro* based on optical density (OD_{600}) for either 4 or 24 h *in vitro* at 30°C, trypan blue staining was used to discriminate between live and dead cells. Note most treatments caused less than 10% cell death after 4 h, whereas, 24 h treatments caused ~40% cell death for all samples except stationary phase supernatant causing 100% mortality (marked by asterisk). Data represent mean ± S.D. of at least 500 per monolayer, three insects/treatment.

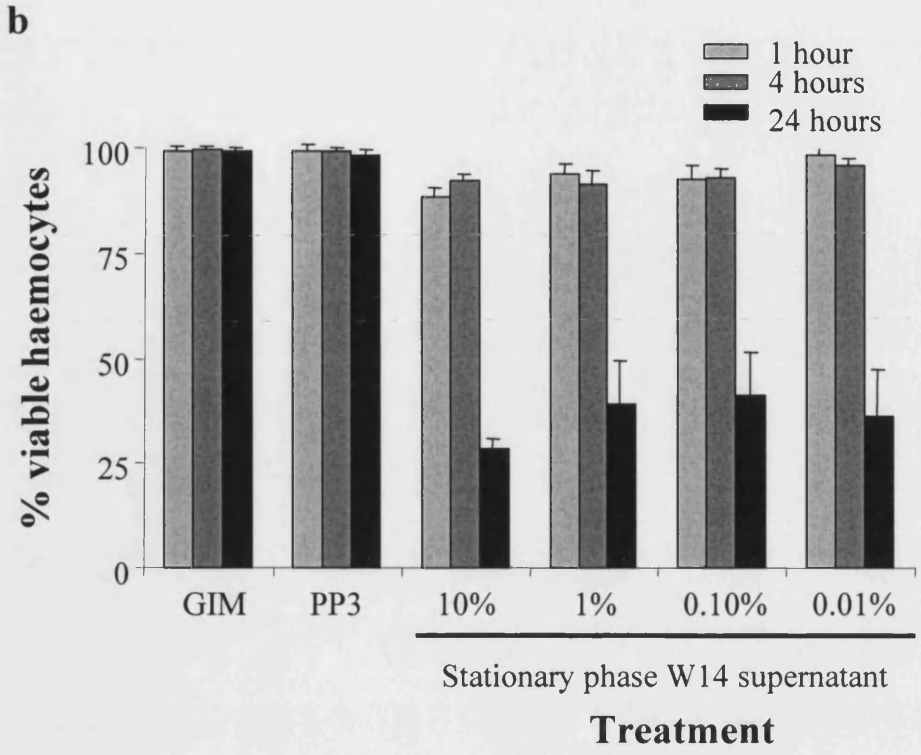

Figure 2.4b Effects of *P. luminescens* W14 stationary phase supernatant on haemocyte viability. Haemocyte monolayers were treated with serial dilutions of stationary phase supernatant previously identified to be cytotoxic against *M. sexta* primary haemocytes *in vitro* (Fig. 2.3 and Fig 2.4a). The monolayers were exposed to the supernatant for 1, 4 or 24 h followed by incubation in 10% PP3 in GIM for a further 23 or 20 and 0 h respectively totalling 24 h *in vitro* at 30°C. The haemocytes were stained with trypan blue and the percentage of viable haemocytes determined. Note that treatments with 1 or 4 h exposure did not cause significant cell death relative to the controls while 24 h treatment resulted in ~ 65% cell death. Interestingly, the cytotoxicity is not affected by the high dilution factor of the supernatant. Data represent mean ± S.D. of at least 500 haemocytes from three monolayers per treatment.

Figure 2.5 Effects of *P. luminescens* W14 supernatant on haemocyte actin cytoskeleton – 4 h *in vitro*. Haemocyte monolayers were treated with either PP3 broth or supernatant from *E. coli*, *Photorhabdus* W14 or K122 at 30°C for 4 h. The treated monolayers were stained with TRITC-phalloidin to highlight the actin cytoskeleton. Typical actin phenotypes for granulocytes (Gr) are shown in Fig 2.5a and plasmatocytes (Pl) in Fig 2.5b and c. Note the polarized actin staining in granulocytes treated with PP3 (a), *E. coli* (d) and *P. temperata* K122 (g), which disappears following treatment with W14 supernatant and is replaced by the presence of microspikes (j). Note also the induction of actin rich stress fibres and dense actin foci in W14 supernatant treated plasmatocytes (k, l), which are absent in the other treatments. (Scale bar = 20μm)

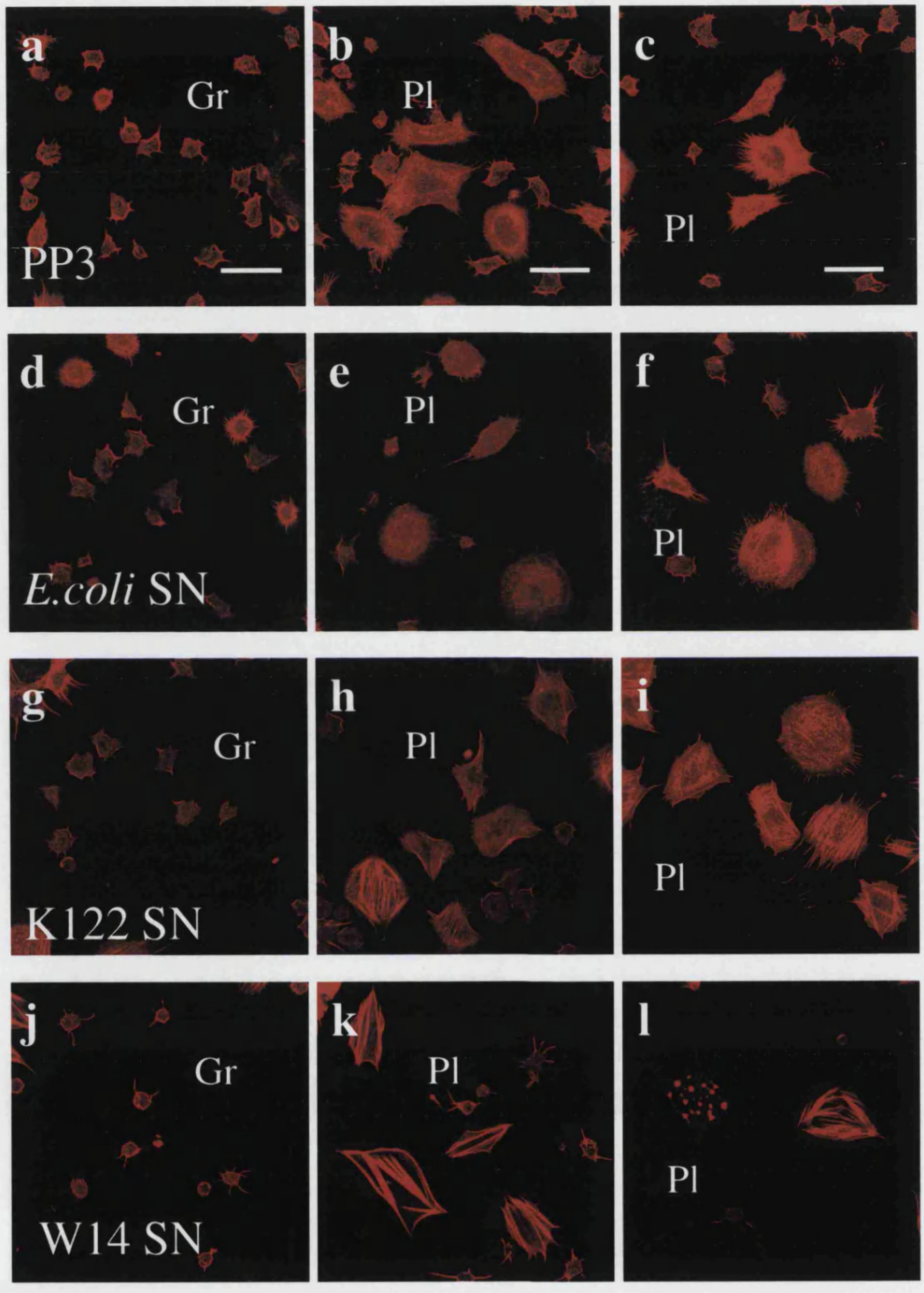
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Figure 2.6 Effects of *Photorhabdus* supernatants on haemocyte actin cytoskeleton – 24 h *in vitro*. Haemocyte monolayers were treated with either PP3 broth or supernatant from *E. coli*, *Photorhabdus* W14 or K122 at 30°C for 24 h. The treated haemocytes were stained with TRIT-phalloidin to visualise the cytoskeleton using confocal microscopy. Note the granulocytes (Gr) treated with in PP3 (a) and *E. coli* supernatant (d) with diffused actin cytoskeleton and less distinct cell polarity relative to the 4 h treatment (Fig. 2.5a). The K122 supernatant treated granulocytes appear shrunken with filopodia protrusions (g) similar to those after W14 supernatant treatment (j). Note also the aggregation of plasmatocytes (Pl) after PP3 (b) and *E. coli* (e) treated plasmatocytes (marked by arrows), which are absent on the monolayers treated with *Photorhabdus* supernatants. K122 supernatant treated plasmatocytes have diffused cytoskeleton and a loss of cell shape (h) while some W14 supernatant treated plasmatocytes display actin foci and others appear constricted with stress fibres throughout cell body (k + l). (Scale bar = 20 μm)

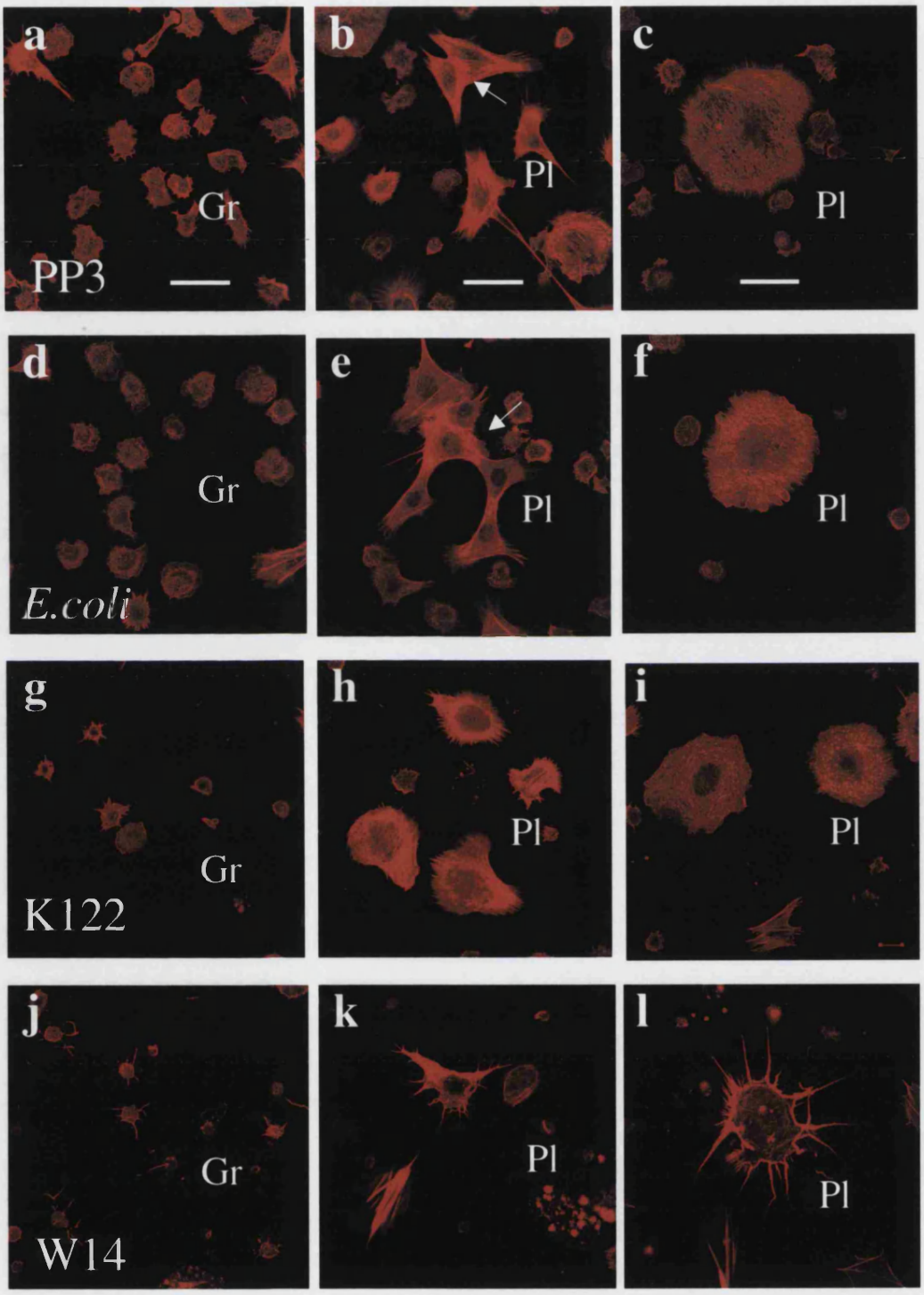
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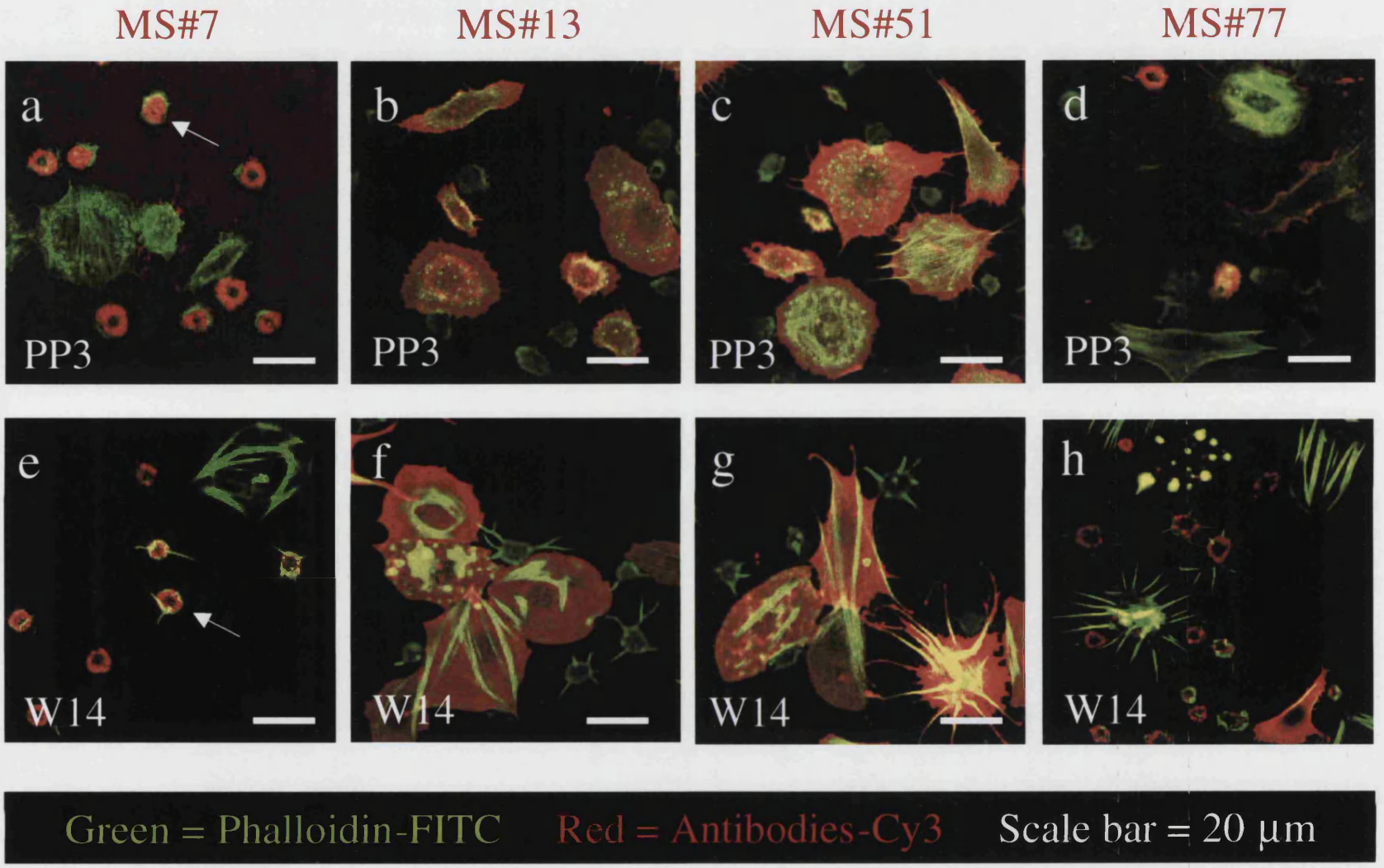
Figure 2.7 Correlation of *P. luminescens* W14 induced actin phenotype to specific plasmatocyte subtypes using immunofluorescence. Several actin phenotypes were observed in plasmatocytes after W14 supernatant treatment, the correlation of W14 supernatant-induced actin phenotypes to plasmatocyte subtypes would indicate the presence of multiple toxins. Haemocyte monolayers were incubated with either PP3 control or W14 supernatant and then haemocytes fixed and immuno-fluorescent labelled with haemocyte subtype specific monoclonal antibodies and a Cy3 secondary antibody (red) and then FITC-phalloidin stained (green). Antibody MS#7 is a positive control which highlights granulocytes specifically show polarised granulocytes after PP3 control treatment (a) and granulocytes with microspikes after W14 supernatant treatment (e). Note MS#13 and MS#51 labelled all plasmatocytes indiscriminately not differentiating the W14 supernatant-induced actin foci / stress fibres and those unaffected (b, c, f, g). Also note MS#77 highlights granulocyte-like round cells with PP3 treatment (d) and labels granulocytes and non-affected plasmatocyte with diffuse actin after W14 supernatant treatment (h). Scale bar = 20 μm


MS#7
MS#13
MS#51
MS#77
a
b
c
d
PP3
PP3
PP3
PP3
e
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W14
W14
W14
W14
Green = Phalloidin-FITC
Red = Antibodies-Cy3
Scale bar = 20 μm

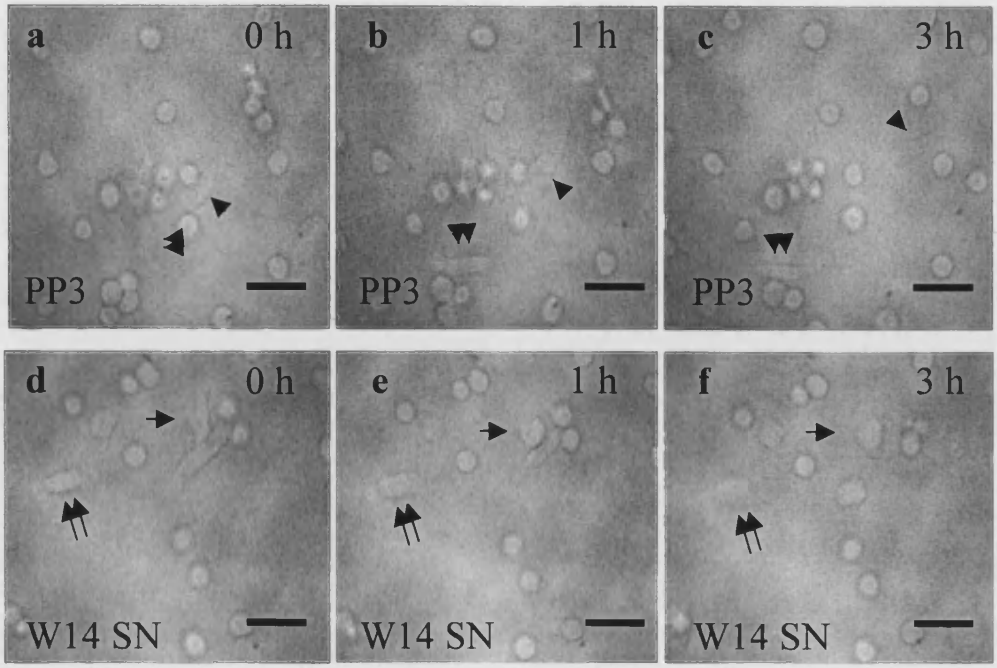


Figure 2.8 Effects of *P. luminescens* W14 supernatant on haemocyte motility. Haemocyte monolayers incubated with either 10% PP3 (a - c) or W14 supernatant (d - f) and viewed under differential interference contrast microscopy. Still images of the same views for each monolayer were recorded at regular intervals over time. Images taken at time 0, 1 and 3 h (before haemocyte death occurs), single and double arrowheads (PP3) and arrows (W14 supernatant) mark the same cell on each monolayer over time. Note the mobility of plasmatocytes with PP3 treatment is inhibited by the presence of W14 supernatant. (Scale bar = 20 μm)

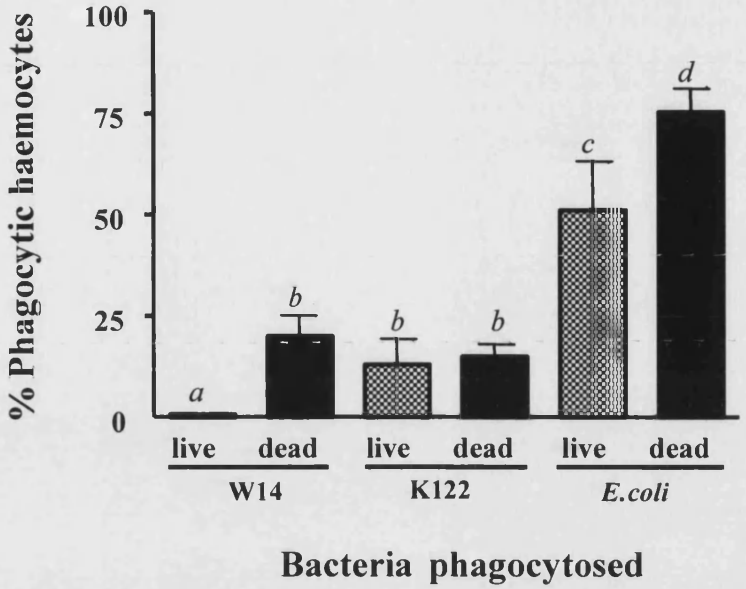


Figure 2.9 Phagocytosis of live or dead *Photorhabdus* or *E. coli* by haemocytes *in vitro* (Au *et al.*, 2004). Haemocyte monolayers were exposed to live or dead (PFA-killed) *Photorhabdus*-GFP or *E. coli*-GFP for 4 h at 30°C to determine the percentage of phagocytic haemocytes. Phalloidin-TRITC stained haemocytes co-localising with GFP-bacteria were scored under confocal microscopy. Both *P. luminescens* W14 and *P. temperata* K122 inhibit their own phagocytosis whether alive or dead. Note the significantly greater effect of the live W14 bacteria over the other *Photorhabdus* treatments. Data represent the mean ± S.E. of at least 500 haemocytes counts per monolayer per insect, three insects/treatment. Means carrying the same letter are not statistically different at the 5% level (paired t-test).

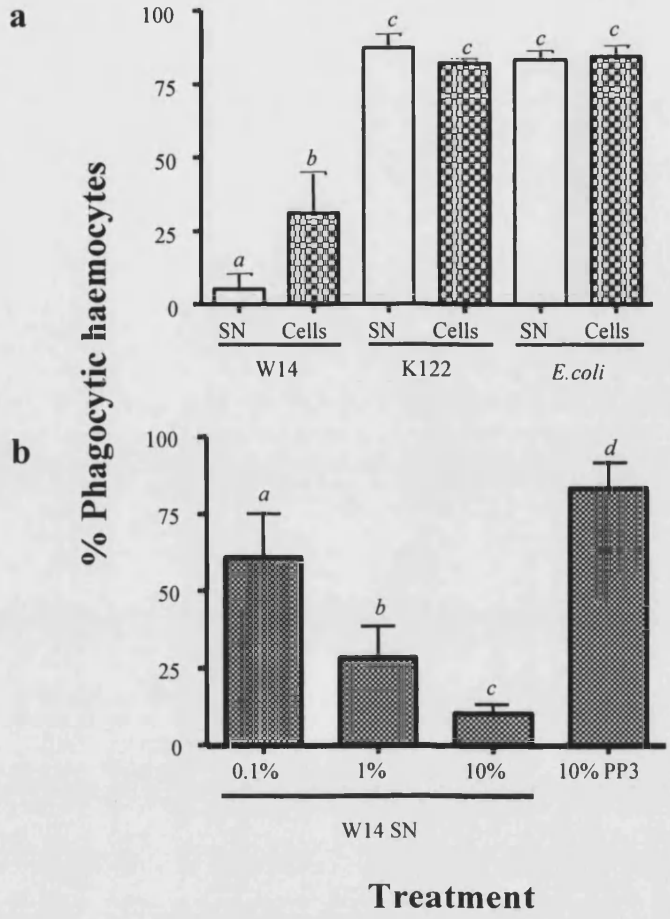


Figure 2.10 Effects of *P. luminescens* W14 supernatant on phagocytic haemocytes *in vitro* (Au *et al.*, 2004). (a) Phagocytosis of *E. coli*-GFP by haemocytes after treatment with cells or supernatant (SN) of either *E. coli* or *Photorhabdus* strains W14 and K122. Haemocyte monolayers were exposed to different treatments and then assessed for their ability to phagocytose *E. coli*-GFP. The percentage phagocytic haemocytes is reduced after treatment with *P. luminescens* W14 cells or supernatant, in contrast, neither fractions of *E. coli* or *P. temperata* K122 had any inhibitory effects. (b) Serial dilutions of the W14 supernatant were examined and concentration-dependent inhibition of phagocytosis was observed. Data represent the mean ± S.E. of at least 500 haemocytes counts per monolayer per insect, three insects/treatment. Means carrying the same letter are not statistically different at the 5% level (paired t-test).

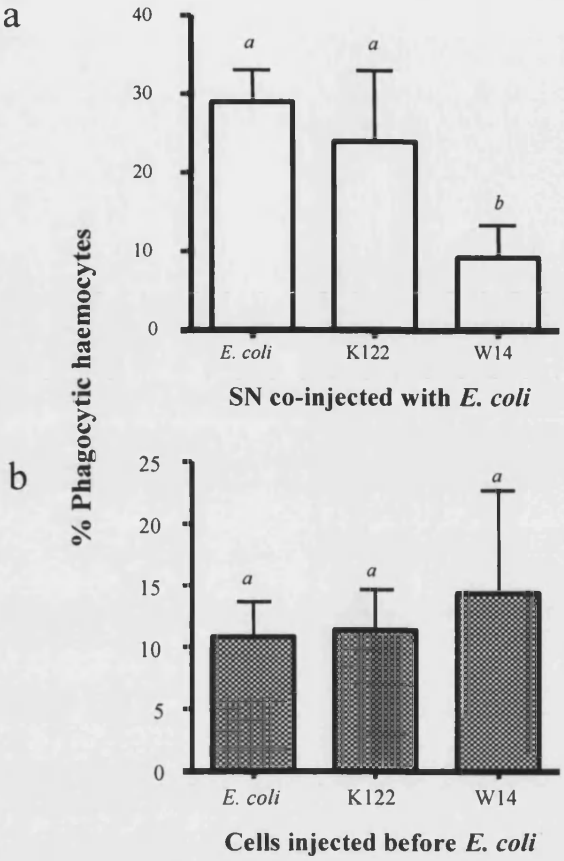
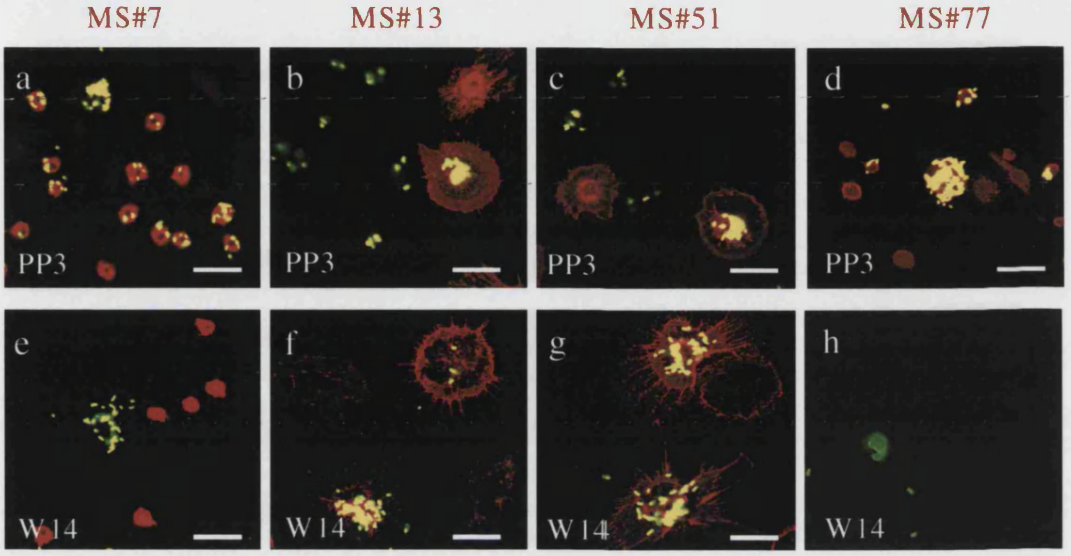


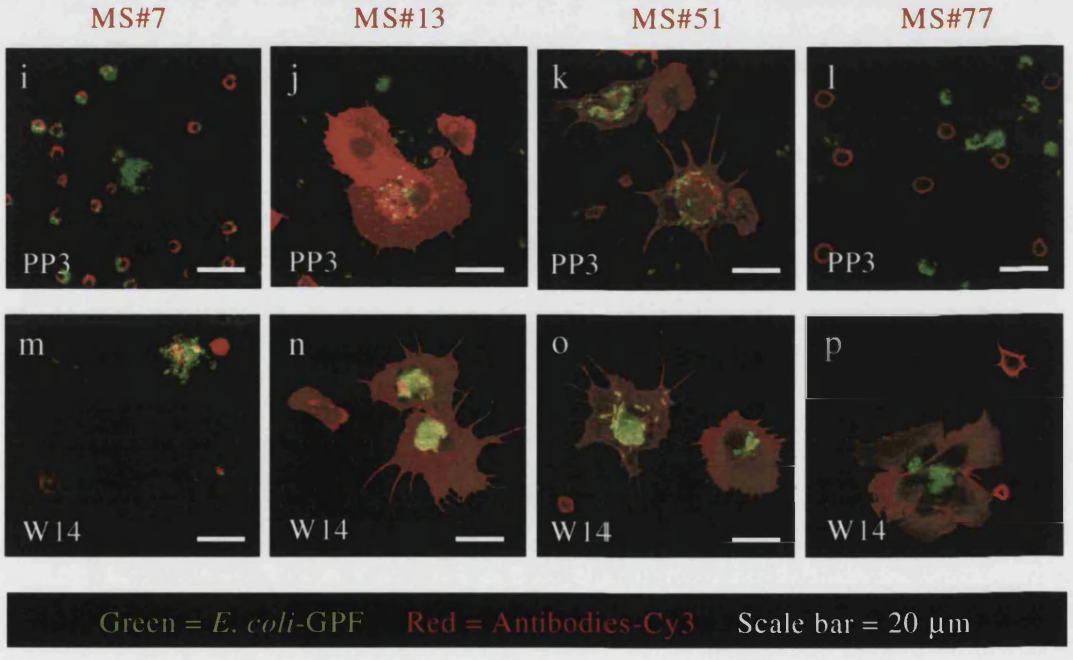
Figure 2.11 Effects of bacterial supernatant or cells on phagocytosis of *E. coli*-GFP by haemocytes *in vivo* (Au *et al.*, 2004). (a) Fifth instar larvae were injected with *E. coli*-GFP suspended in the supernatant of *E. coli*, *Photorhabdus* W14 or K122, to assess the effects on haemocyte phagocytosis *in vivo*. Counts from monolayers prepared with haemolymph extracted 2 h post-injection revealed W14 supernatant co-injected with *E. coli*-GFP reduced the percentage of phagocytic haemocytes. (b) Larvae injected with 1 x 10^5 cells of either *E. coli* or *Photorhabdus* W14 or K122 as pre-treatment and incubated at 25°C for 2 h. Each larva was then injected with 1 x 10^7 cells of *E. coli*-GFP, incubated for a further 2 h, haemolymph was extracted from the larvae and monolayers prepared to determine the percentage of phagocytic haemocytes present. None of the bacteria initially injected significantly altered the percentage of phagocytic haemocytes observed on the monolayers. Data represent the mean + S.E. of at least 500 haemocytes counted per monolayer, three insects per treatment. Means carrying the same letter are not statistically different at the 5% level (paired t-test).

Figure 2.12 Phagocytic haemocyte populations of *M. sexta in vitro* and *in vivo*. (Top) *In vitro* experiments required haemocyte monolayers prepared from naive larval haemolymph which were subsequently exposed to *E. coli*-GFP with either PP3 control (a, b, c, d) or W14 supernatant (e, f, g, h). Confocal analysis revealed granulocytes, hyperphagocytes and plasmatocytes in order of abundance, make up the phagocytic haemocyte population *in vitro*. Note that *P. luminescens* W14 supernatant inhibits phagocytosis by granulocytes, to which the hyperphagocytes appear resistant. (Bottom) For *in vivo* experiments, *M. sexta* larvae were injected with *E. coli*-GFP suspended in either PP3 (i, j, k, l) or *P. luminescens* W14 supernatant (m, n, o, p) and bled 2 h post-injection. Monolayers prepared from the haemolymph were labelled with haemocyte antibodies and a Cy3-conjugated secondary antibody (red), followed by FITC-phalloidin staining (green). Note granulocytes, hyperphagocytes and plasmatocytes make up the phagocytic population *in vivo* as is observed *in vitro*, again only granulocytes are inhibited by W14 supernatant. Also note MS#7 and MS#77 labelling targets were altered by W14 supernatant treatment (see text).

In vitro



In vivo



Green = *E. coli*-GPF Red = Antibodies-Cy3 Scale bar = 20 µm

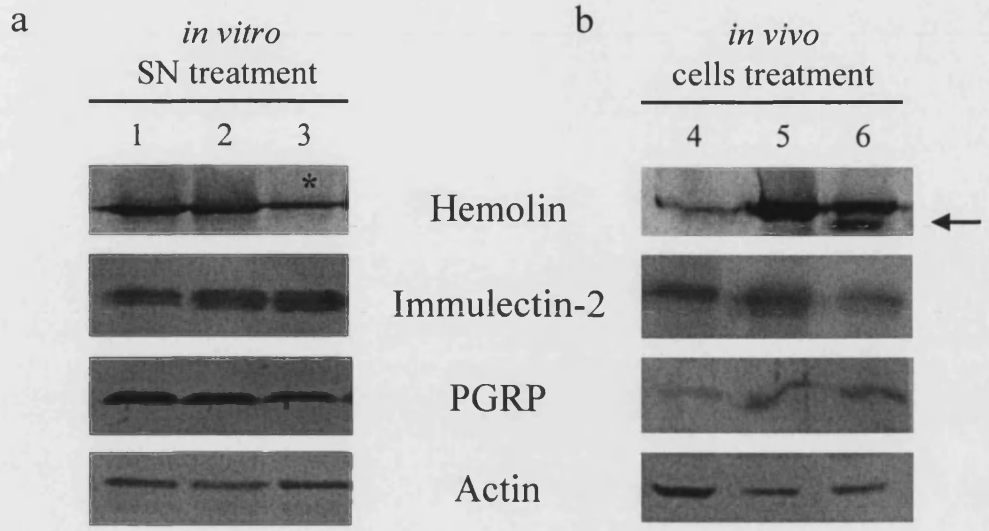


Figure 2.13 Western analysis of *M. sexta* immune recognition molecules post bacterial challenge *in vitro* and *in vivo*. (a) Haemolymph from naive larvae was incubated *in vitro* with (1) GIM, (2) *E. coli* supernatant or (3) W14 supernatant at 25°C for 12 h. Extracts from the individual blots show positive signals against hemolin, immulectin-2 and peptidoglycan recognition protein (PGRP) and actin. Note the slightly reduced intensity of hemolin signal in the W14 supernatant treated sample indicated by asterisk (*). (b) *M. sexta* larvae were injected with (4) GIM, (5) *E. coli* cells or (6) W14 cells and incubated at 25°C for 24 h. Haemolymph was extracted from each larva and immuno-blotted against the recognition molecule anti-bodies. Extracts of individual blots show both hemolin and PGRP were induced by the *in vivo* bacterial challenge. Note the extra band against hemolin in the W14 injected haemolymph indicated by arrow. Some non-specific bands were visible on the blots, extracts taken from the individual blots were at the appropriate size for each protein; hemolin at 47 kDa, immulectin-2 at 36 / 37.5 kDa, PGRP at 19 kDa and actin at 42 kDa.

2.4 Discussion

While significant effort has been made to characterise specific virulence factors from different strains of *Photorhabdus*, as yet the response of insect immune system to a normal *Photorhabdus* infection has received little attention. Previous work on *P. luminescens* W14 revealed cell-associated and secreted factors which alter *M. sexta* haemocyte immune functions (ffrench-Constant *et al.*, 2003; Dean, 2002; Silva *et al.*, 2002). Brillard *et al.* (2001) report the presence of two distinct cytotoxins in the supernatant of *X. nematophila* against *Spodoptera littorallis* haemocytes and mammalian red blood cells. Further, the authors report the absence of such cytotoxins in the supernatants of isolates belonging to various *Photorhabdus* species, however *P. luminescens* W14 was not included in their study.

Described here is a combination of *in vitro* and *in vivo* assays to demonstrate the presence of both cell-associated and secreted cytotoxic factors in *Photorhabdus* strains against *M. sexta* haemocytes, in comparison to that of a non-pathogenic *E. coli*. The chosen *Photorhabdus* strains have distant phylogenic relationships as determined by two independent analyses (Marokhazi *et al.*, 2003; Szallas *et al.*, 1997), encouraging the speculation that the two *Photorhabdus* strains employ distinct strategies to overcome the insect host immune responses.

To compare the infection patterns of *Photorhabdus* strains W14 and K122 and a non-pathogenic *E. coli*, *M. sexta* larvae were injected with either *E. coli* or *Photorhabdus* strains, and then bled over time to monitor the recoverable bacterial count and total haemocyte count. As expected all *E. coli* was cleared from the insect haemolymph rapidly and provoked a transient increase in total haemocyte count, which recovered promptly to resting level as previously observed (Dunn and Drake, 1983; Horohov and Dunn, 1982). This may reflect an active host immune system response to infection by increasing the number of circulating phagocytes to effectively remove bacteria from the haemolymph. In contrast to *E. coli*, both *Photorhabdus* strains grew unrestricted in the insect haemocoel accompanied by dramatic decrease in total haemocyte count prior to insect death. Interestingly, *P. temperata* K122 caused an immediate reduction in haemocyte count and appeared to

begin replication in the haemocoel without delay, while *P. luminescens* W14 caused a gradual reduction in haemocyte count and displayed an initial lag period of about 12 h before growth in the haemocoel was detectable. Silva *et al.* (2002) reported the association of W14 bacteria to the insect larval midgut, which may provide a niche for W14 bacteria to reside immediately upon entry into insect haemocoel, where they may escape haemocyte contact and replicate undetected. These differences between the infection patterns suggest distinct modes of virulence for the two pathogens, which is in accordance with previous suggestion that *Xenorhabdus* and *Photorhabdus* employ different pathogenic strategies to complement that of their nematode partners (Rosa *et al.*, 2002; Forst *et al.*, 1997).

To examine the interaction between bacteria and haemocytes and also the effects of bacterial supernatants on haemocytes, haemocyte monolayers were exposed to bacterial cells and supernatant of *E. coli*, *Photorhabdus* W14 or K122 *in vitro* and then examined using the light microscope. Data revealed both *P. luminescens* W14 and *P. temperata* K122 cells remained attached to haemocytes, in contrast to *E. coli* cells that were absent from monolayers, presumably phagocytosed by surrounding haemocytes (Fig. 2.2). The ability to avoid phagocytosis while remaining attached to host cells is seen in other Gram-negative pathogens such as *Yersinia* species (Allen, 2003; Fallman *et al.*, 1995) and EPEC (Nougayrede *et al.*, 2003) and has been previously reported for *P. luminescens* W14 (ffrench-Constant *et al.*, 2003). Such interaction requires the bacteria to utilise attachment molecules and Type III secretion system (TTSS), *P. luminescens* W14 sample sequence data available indeed confirm the presence of *invasin*, *ail* and components of the TTSS (ffrench-Constant *et al.*, 2003), as well as multiple pilus operons found in the recently published TTO1 genome (Duchaud *et al.*, 2003) which may allow such bacteria-host cell interaction. It would be interesting to investigate these loci with targeted mutants and monitor any disruptions to the interaction with haemocytes. Unfortunately, targeted knockout technology is not established in W14 due to general difficulties in genetic manipulation (see section 5.4). Alternatively, exogenous expression in *E. coli* may shed some light on the function(s) of these genes, however, attempts in Ail expression were not successful using recombinant *E.*

coli clones carrying *ail*^{W14} plasmid construct. Hence, the ability of the recombinant clone to persist in the larval haemocoel could not be assessed.

P. luminescens W14 supernatant induced changes in haemocyte morphology visible under the light microscope, haemocytes displayed more prominent nuclei and appeared spread with larger cell surface areas. The viability of the haemocytes treated with bacterial supernatants was determined over a period of 24 h. Both K122 and W14 supernatants caused haemocyte death, but W14 did so after only 4 h while K122 induced haemocyte death after 8 h incubation. This difference in kinetics could be due to different toxins present with differential modes of action (Finlay and Falkow, 1997; Rowe and Welch, 1994). A closer study of W14 supernatant samples from various growth phase tested revealed only stationary phase W14 supernatant cause high percentage mortality after 24 h incubation. Interestingly, W14 stationary phase supernatant required prolonged exposure to haemocytes of more than 4 h to achieve greater than 10 % mortality and mortality caused by this sample was not concentration-dependent. One possible explanation of this is that cytotoxicity may be caused by toxin(s) present in abundance, hence cytotoxicity was sustained despite dilution by three orders of magnitude. Alternatively, enzymatic factor(s) may be present allowing recycling of the toxin(s). Together these data suggest W14 and K122 have cell-associated cytotoxicity while W14 also has secreted factors in its supernatant that are toxic toward *M. sexta* haemocytes.

Prime candidates for cytolytic activity are the loci with homology to *Serratia marcescens shlBA* (Hertle, 2000) and *P. luminescens* subsp. *laumondii* strain TT01 *phlBA* operon (Brillard *et al.*, 2002), however, unsuccessful cloning of these genes prevented further investigations. A similar actin phenotype of condensed actin foci was observed in haemocytes treated with Mcf1 toxin from *P. luminescens* W14 (see Chapter 4). The mechanism of action for Mcf1 is currently under investigation but it is believed to induce apoptotic phenotypes in infected *M. sexta* larvae midgut epithelial cells *in vivo* (Daborn *et al.*, 2002), Mcf1 also caused membrane blebbing of *M. sexta* haemocytes *in vitro* (Daborn *et al.*, 2002) and apoptosis in mammalian culture cell-lines (Dowling *et al.*, 2004). These observations make Mcf1 a candidate toxin during an infection, however, no evidence of its expression *in vivo* is available.

Haemocytes exposed to bacterial supernatants labelled with fluorescein-phalloidin allowed a closer examination of the cells actin morphology. Confocal analysis revealed that *P. luminescens* W14 supernatant causes actin rearrangements in haemocytes. *P. luminescens* W14 supernatant treated granulocytes lost their polarity and produced microspikes, which protruded from shrunken cell bodies and plasmatocytes lost the diffuse cytoplasm and gained stress fibres, actin cables or condensed actin foci throughout the cell bodies. In contrast, neither *E. coli* nor *P. temperata* K122 supernatant treatments induced such actin phenotypes after 4 h *in vitro* and *P. temperata* K122 supernatant caused less dramatic actin rearrangements but only after 24 h incubations, again emphasising the difference between W14 and K122 discussed previously. As the W14-induced actin rearrangements precede cell death, the disruption of cellular actin dynamics could have been the direct cause of cell death. Additionally, haemocytes exposed to PP3 control or *E. coli* supernatant were seen to aggregate on the coverslip after 24 h incubation, a behaviour which was not evident on monolayers treated with either of the *Photorhabdus* supernatants. This *in vitro* formation of microaggregates is believed to represent the early stages of the nodule formation that occurs *in vivo* in response to the presence of microbes and microbial pattern molecules (Miller and Stanley, 2001; Stanley *et al.*, 1998). Microaggregate formation *in* vitro is dependent on cell movement, hence the motility of haemocytes was therefore examined using differential interference contrast (D.I.C.) time-lapse microscopy, and W14 supernatant treatment did indeed inhibit haemocyte motility. Therefore, the *Photorhabdus* strains may actively disrupt haemocyte actin dynamics and subsequently diminish haemocyte functions including phagocytosis (Dean, 2002; Silva *et al.*, 2002) and nodulation (Dean, 2002).

Actin dynamics are essential to the survival of all organisms and unsurprisingly, many bacterial toxins exploit the actin cytoskeleton in target host cells (Barbieri *et al.*, 2002; also see section 1.3.3.4). Potential toxin targets include actin itself as well as components in signalling and regulatory pathways (section 1.2.5.2). The loss of granulocyte polarity and gain of stress fibres in plasmatocytes may be caused by modulation in activity of the Rho family of GTP binding proteins (Etienne-Manneville and Hall, 2003). The *P. luminescens* W14 sample sequence analysis has revealed that within the genome are homologues of known toxins such as ADP-

ribosyltransterases, cytotoxic necrosis factors, *Yersinia* Type III delivered effecter proteins and Rtx toxins, which could be responsible for the observed actin rearrangements (ffrench-Constant *et al.*, 2000). To test this hypothesis, cosmid library clones of *P. luminescens* W14 containing toxin candidates; *rtx-A* open reading frame, *rtx*-like operon, *E. coli* macrophage toxin-like and CNF-like locus were selected and tested for wildtype *P. luminescens* W14 supernatant-induced effects on haemocytes. However, when the supernatants of these *E. coli* clones containing candidate toxins were overlaid on to haemocyte monolayers, none induced haemocyte death or actin rearrangements, as seen with wildtype *P. luminescens* W14 stationary phase supernatant treatment. These results could have been the consequence of little or no expression of the candidate gene products. Subsequent attempts to clone the loci individually for exogenous expression studies were not successful.

As phagocytosis is vital to the role of professional immune cells, the phagocytic competence of the haemocytes was examined using a combination of *in vitro* and *in vivo* assays. The data show both live and dead *Photorhabdus* W14 and K122 cells are able to suppress their own phagocytosis *in vitro*. This inhibition is assumed to be the case *in vivo* as both strains are able to grow uninhibited in *M. sexta* (Fig. 2.1a). Only *P. luminescens* W14 cells and supernatant reduced the haemocytes ability to phagocytose *E. coli in vitro* while neither fractions of *P. temperata* K122 had any effects. Intriguingly, the anti-phagocytic activity is concentration-dependent implying that the concentration-independent cytotoxic factor(s) present in the supernatant are unlikely to be responsible for the suppression of phagocytosis. Interestingly, while *P. temperata* K122 suppresses its own phagocytosis *in vitro*, it does not inhibit that of *E. coli*, again demonstrating that it uses a different virulence strategy to that of *P. luminescens* W14. Restricted suppression of phagocytosis would be an advantage *in vivo*, as the insect haemocytes would continue their surveillance against other microbial invaders, reducing potential competition in the potential host. The production of multiple anti-microbial compounds by *Photorhabdus* strains (Derzelle *et al.*, 2002; Sharma *et al.*, 2002; Bondi *et al.*, 1999; Li *et al.*, 1995) does indeed suggest that competition for insect resources is a potential problem.

When the bacterial cells and supernatants were examined for their effects on phagocytosis *in vivo*, *P. luminescens* W14 supernatant was shown to suppress the phagocytosis of *E. coli*, while K122 and *E. coli* supernatants did not. Despite this, pre-injection with W14 cells failed to suppress *in vivo* phagocytosis of *E. coli*. This was unexpected, however, closer analysis of the raw data revealed that although the percentage of phagocytic haemocytes in the W14 injected treatment was similar to that of the controls, the actual numbers of adhered haemocyte count per random view were strikingly different between the two treatments. It was necessary to examine more random views to compensate for the lower haemocyte counts. It is likely, therefore, that the general reduction of adherent haemocytes in the *Photorhabdus* pre-treated larvae had resulted in a biased count. That is, the phagocytes' inherent ability to adhere might have resulted in a false representation (an increase) in the percentage of active phagocytes relative to a lower total haemocyte count. This particular problem could be solved be using fluorescence-activated cell sorting (FACS) as the method of cell count as the method of haemocyte collection does not depend of the haemocyte adhesiveness.

Morphological differences imply existence of sub-types amongst the haemocyte population, however, as haemocyte behaviour and hence morphology vary according to environmental factors, tools such as haemocyte specific monoclonal antibodies (mAbs) allow more reliable method of differentiation (Trenczek, 1998; Willott *et al.*, 1994). Immuno-fluorescence analysis revealed that MS#13 stained all haemocytes except granulocytes on monolayers *in vitro* (Fig. 2.12). Both phagocytic and non-phagocytic plasmatocytes stained positively against MS#13. This antibody (mAb MS13) has been shown to inhibit plasmatocyte spreading *in vitro* and also inhibits encapsulation of latex beads *in vivo* and *in vitro* (Wiegand *et al.*, 2000). This mAb MS13 is reported to recognise a 90 kDa plasmatocyte surface protein that is likely to mediate adherence, spreading and therefore possibly encapsulation (Wiegand *et al.*, 2000). Data from the present study show this 90 kDa protein to be present on the surface of both phagocytic and non-phagocytic plasmatocytes, inferring the protein unlikely to be directly involved in the phagocytic process. Although this speculation is not in accordance with the observed inhibition of *in vitro* spreading (Wiegand *et al.*, 2000), however, it may have roles in haemocyte-haemocyte interactions that are

required for the multi-cellular encapsulation process (Gupta, 1991). Overall, the use of available mAbs in these studies unfortunately did not differentiate plasmatocyte sub-types and so correlation between plasmatocyte subtypes and specific actin phenotypes was not possible.

Confocal microscopic observations also showed the antibody MS#77 to label different haemocyte subtypes with *P. luminescens* W14 supernatant treatment compared to control. This suggests that exposure to *P. luminescens* W14 supernatant may result in altered haemocyte surface protein profile, which may reflect *Photorhabdus*-induced changes in host cell surface components by candidate proteins such as the Rtx-like metalloprotease (Bowen *et al.*, 2002). Alternatively, these changes in haemocyte surface components could be a natural course of cellular changes in response to detection of soluble bacteria material. Characterisation of the nature of these changes may lead to identification of important immune haemocyte receptors targeted by the pathogens to ensure the bacterial persistence. Microarray analysis of transcriptional changes in cultured *Drosophila* haemocytes is a powerful method to address a complex problem such as this, which is currently underway in our laboratory. Indeed, a *Spodoptera* microarray is under construction (Rene Feyereisen, personal communication), which could be used to monitor changes in cultured SF9 cells. This would provide a lepidopteran model, more closely related to *M. sexta.*

Nevertheless, these immuno-fluorescence studies did reveal that granulocytes are susceptible to inhibition of phagocytosis by *P. luminescens* W14 supernatant *in vitro* and *in vivo*, and also demonstrated that phagocytosis by hyperphagocytes is unaffected by the *Photorhabdus* supernatants. This raises intriguing questions over how the bacteria use their secreted factors to influence the interaction with particular host tissue types? Are hyperphagocytes resistant to secreted *Photorhabdus* toxins or targeted by *Photorhabdus* specifically for intracellular residence? Do intracellular conditions in hyperphagocytes permit *Photorhabdus* to grow and replicate? M cells are terminally differentiated epithelial cells specialises in sampling of intestine luminal material and presentation of foreign bodies to underlying macrophages (Siebers and Finlay, 1996). M cells exhibit reduced mucus layer, shortened

microvilli, and relatively high endocytic and pinocytic uptake in comparison to columnar epithelial cells (Finlay and Falkow, 1997). These unique properties constitute a target for many enteric pathogens to breach the mucosal barrier in the small intestine (Siebers and Finlay, 1996). Further investigation on the nature of the hyperphagocytes (described by Dean, 2002) and their interaction with *Photorhabdus*, *E. coli* and abiotic substrates could result in advances in the knowledge of pathogenic strategies as well as cellular immunity at molecular level.

Finally, Western analyses were carried out to monitor the presence of selected recognition molecules, and to determine if they are targeted by *P. luminescens* W14 toxins. The data did not conclusively reveal any reduction of the proteins tested, although an extra band cross-reacting with the anti-hemolin antibody did appear in the *P. luminescens* W14 injected larval haemolymph. This is suggestive of molecular alteration of hemolin, causing a size reduction, however, quantitative methods such as ELISA would be necessary to make a more precise assessment. Again, any immune molecules actively disrupted by pathogens would indicate their importance in the insect immune responses. Collectively, the level of these infection-induced proteins appeared equal between *E. coli*- and *Photorhabdus*-infection injected larvae, implying that *Photorhabdus* is detected by the insect immune system. Although in principle, the observed increase in hemolin and PGRP levels could have been a result of wounding by injection alone, since a sham injected control that was omitted from these studies. Subsequent work at Bath has shown that such wounding does not alter the expression of these genes in *M. sexta* (Reynolds, person communications)

To summarise, this chapter documents alterations of several haemocyte phenotypic and functional characteristics when exposed pathogenic *Photorhabdus* fractions using *in vivo* and *in vitro* assays. Both *Photorhabdus* K122 and W14 have cell-associated and secreted toxins active against *M. sexta* haemocytes, although the toxins present and their modes of action as well as the overall infection strategy appear to be different. The documented characteristics form the basis for development of screening methods to isolate the toxins responsible. Knowledge of the "normal" responses will also allow a better understanding of any changes in the

host-pathogen interaction seen in targeted *Photorhabdus* mutants. The host molecule targets of *Photorhabdus* virulence factors will shed light on cellular components important in the insect immune system.

Chapter 3

Effects of orally toxic Toxin complexes on haemocytes

3.1 Introduction

Oral toxicity is a characteristic recently identified in a few *Photorhabdus* isolates (Marokhazi *et al.*, 2003) and first described in *P. luminescens* W14 (Bowen *et al.*, 1998). As mentioned in the introduction (section 1.1.4.1), these large molecular-weight toxin complexes (Tcs) account for oral toxicity and therefore are potential candidates as insecticidal transgenes in crops as biological pest control (Bowen *et al.*, 1998). The Tc toxins provide an alternative to conquer concerns over the evolution of insect resistance to the currently deployed *Bacillus thuringiensis* δ-endotoxins (ffrench-Constant and Bowen, 2000).

Since the identification of the four *tc* loci in *P. luminescens* W14, successful purification of the toxin complexes from *P. luminescens* W14 culture supernatant has enabled development of Tc-specific antibodies (Bowen and Ensign, 1998) and molecular cloning of the gene loci; *tca*, *tcb*, *tcc* and *tcd* (Bowen *et al.*, 1998). The disruption of *tc* loci in *P. luminescens* W14 revealed *tca* and *tcd* to account for the majority of the oral toxicity of *P. luminescens* W14 against *M. sexta* larvae (Bowen *et al.*, 1998). This is supported by recent microarray analysis where the *tca* loci was correlated perfectly in all *Photorhabdus* isolates with orally toxic supernatant, while *tcd* was related to bacterial cell-associated oral toxicity (Marokhazi *et al.*, 2003). The original studies by Bowen *et al.* (1998) also showed that although *tcb* and *tcc* are not necessary for oral toxicity, they do make a minor contribution, implying complex interactions between the toxin complex components. The *Tc* toxins' modes of action are unknown although the midgut epithelium has been identified as a target, alternative targets such has the haemocytes have also been suggested (ffrench-Constant *et al.*, 2003; Waterfield *et al.*, 2001b).

Histopathology studies of Tca-treated *M. sexta* showed that *tca*-encoded products cause disintegration of larval midgut when administered orally or injected directly into the haemocoel (Blackburn *et al.*, 1998). These observations raise questions over the target specificity of the Tca and how it causes similar effects on the gut epithelium whether acting from the gut lumen or from the haemocoel side. Moreover, the midgut histopathology observed with Tca treatment is similar to that of wildtype *P. luminescens* W14 treatment, as well as that of *B. thuringiensis* endotoxins (pore-formers; Vie *et al.*, 2001) and cholesterol oxidase (attacks target cell membrane lipids; Purcell *et al.*, 1993). Therefore the blebbing of the insect midgut epithelial cells is likely to be a common downstream effect rather than due to shared initial mode of action (ffrench-Constant and Bowen, 2000).

In the search for the toxic component of the Tcs, Bowen and colleagues (2000) demonstrated that the three proteases co-purified with the Tc toxins are not necessary for oral toxicity against *M. sexta.* Diligent investigation led to the discovery that three basic components; TcdA, TcdB and TccC, confer oral toxicity when expressed in *E. coli* (Waterfield *et al.*, 2001a). Interestingly, it was demonstrated that only TcdA and TcdB are required to confer oral-toxicity when these genes are expressed in the normally non-orally toxic *P. temperata* K122, while the additional expression of TccC is necessary to confer oral toxicity when these genes are expressed in *E. coli.* Transmission electron microscopy showed TcdAB to form ball-stick 'lollipop' structures, which were not altered by the addition of TccC. These data suggest that *tccC* encodes either an active toxin itself or an accessory protein such as a chaperone or an activator, which is already present in K122 (Waterfield *et al.*, 2001a).

The constantly increasing availability of genomic sequence data has accelerated the understanding of Tc toxins. Sequence analysis of *tc* loci and other associated regions has enabled the description of pathogenicity islands in *P. luminescens* W14 consisting of many other potential toxins (Waterfield *et al.*, 2002b) and aided the identification of *tc* homologues in another insect pathogen, *Serratia entomophila* and a human pathogen *Yersinia pestis* (Waterfield *et al.*, 2001b) adding to confusion over their likely biological functions. The phylogenetic analysis of *tccC*-like sequences from these diverse bacteria and other *Photorhabdus* and *Xenorhabdus* strains has led

to the proposals that the *tc* loci are derived from an ancient progenitor that predates the split of Gram-positive and Gram-negative bacteria (Waterfield *et al.*, 2001b). Further, *tc*-associated loci such as those encoding phage-related proteins imply cell lysis as a possible method of releasing the large Tc toxins.

Most interestingly, homology of *tc* loci to other known bacterial toxins could provide clues to the Tc toxins' biological functions, particularly with regards to possible roles in interaction with haemocytes. Of interest are regions in *tcaB, tccB* and *tcdA* bearing homology to each other and to the *Salmonella* plasmid-borne virulence factor (Spv) proteins. The *S. dublin* SpvA (Libby *et al.*, 2002) and *S. typhimurium* SpvB (Otto *et al.*, 2000) were shown to be involved in macrophage-interaction in their natural hosts. Moreover, the SpvB carboxyl terminus was shown by Otto and colleagues to be an ADP-ribosyltransferase implying possible roles in modulation of cellular actin structure as observed in chapter 2.

While the above reviewed research provides clues to the Tc toxins' likely mode of action, intriguing questions regarding the active toxin(s) identity, target specificity, toxin components stoichiometry, *in vivo* expression pattern and secretion pathway remain unanswered. Despite the lack of complete understanding, Liu *et al.* (2003) have realised the potential of Tcs for crop protection purposes and demonstrated that high level expression of a *P. luminescens* W14 TcdA transgene in *Arabidopsis thaliana* confers toxicity against *M. sexta* larvae on the host plant (Liu *et al.*, 2003).

In this chapter, I describe experiments that were designed to investigate possible effects of Tcs on *M. sexta* haemocytes, with particular attention to the phenotypes previous observed in haemocytes treated with Tc-containing *P. luminescens* W14 supernatant. *tc*-knockout mutants of *P. luminescens* W14 as well as recombinant expression of *tc* components in *P. temperata* K122 and *E. coli* help to identify Tcs involved in the disruption of the insect host immune-competent cells.

3.2 Materials and method

3.2.1 Insects and bacterial stains

M. sexta were reared as described in section 2.2.1. *P. luminescens* W14 wildtype and *tc* knockout (*tc*-) mutant strains (kind gifts from Dr. Mark Blight, France) were stored as glycerol stocks at -80°C streaked onto 2% PP3 agar plates with the appropriate antibiotics; *P. luminescens* W14 wildtype – none, *tca*- and *tcb*- with 10 μg ml^{-1} streptomycin, *tcc*- with 25 μg ml^{-1} chloramphenicol, *tcd*- with 50 μg ml^{-1} kanamycin and *tca-/tcd*- with 10 μg ml^{-1} streptomycin and 50 μg ml^{-1} kanamycin and incubated at 30°C for 48 h. Fresh colony picks from plates were used to inoculate 100 ml of PP3 broth supplemented with antibiotics stated. The cultures were grown aerated at 30°C for 48 h then centrifuged to pellet bacteria and the decanted supernatant samples were filter sterilised through 0.2 μm syringe driven filters (Millipore) and stored at 4°C until use.

P. temperata K122 (isolated by Dr. D. Clarke, Republic of Ireland) and recombinant K122 carrying plasmids; [tca^{W14}] consists of the W14 loci *tcaA*, *tcaB* and *tcaC* in Bluescript pBCKS(+) and [tcd^{W14}] with W14 *tcdA* and *tcdB* in pBR322 and [$tcd+tccC^{W14}$] with an additional *tccC* open reading frame cloned into the [tcd^{W14}] plasmid backbone (constructed by Dr. N. Waterfield, University of Bath, U.K.). These were stored in glycerol stock in –80°C until use. Fresh plate colonies from LB agar plates were used to inoculate 100 ml LB broth with the appropriate antibiotics; K122 wildtype – none, K122 [tca^{W14}] – 25 μg ml^{-1} of chloramphenicol and K122 [tcd^{W14}] and K122 [$tcd+tccC^{W14}$] - 100 μg ml^{-1} of ampicillin. Cultures were grown at 30°C for 48 h and cell-free supernatant extracted as above.

E. coli ± [tcb^{W14}] was grown in LB supplemented with 100 μg ml^{-1} ampicillin, aerated at 37°C with samples taken at 12, 24 and 48 h post-inoculation. Supernatant sample were extracted as described above, the cell pellets were retained and sonicated as described in section 5.2.6 to obtain cell lysate samples.

3.2.2 Oral bioassays

First instar *M. sexta* neonates were placed and fed on ~1 cm^3 discs of artificial wheat germ diet laced with 100 µl filtered sterilised supernatants then air dried. The larvae were incubated at 25°C for 72 h and weighed individually to monitor weight gain and to score for larval mortality. Three larvae per treatment were used in three independent experiments.

3.2.3 *In vitro* haemocyte mortality assay

P. temperata K122 wildtype, and recombinant K122 with plasmids [tca^{W14}], [tcd^{W14}] and [tcd+$tccC^{W14}$], *P. luminescens* W14 wildtype and *tc*- mutants were grown and supernatant extracted as described in section 3.2.1.

Haemocyte monolayer preparations and mortality assay procedures were carried out as described previously in section 2.2.8. Data shown were calculated from counts of at least 500 cells / monolayer / insect and three insects per treatment.

3.2.4 Haemocyte actin staining and confocal analysis *in vitro*

P. luminescens W14 supernatant were prepared as described in section 3.2.1. The particulate toxin complex preparation protocol is as described by Waterfield *et al.*, 2001 (kindly prepared by A. Dowling, University of Bath, U.K.). Bradford assays (Bio-rad) were performed to estimate the protein content of the preparations, final concentration of 0.75 mg ml^{-1}. Fluorescein-conjugated phalloidin labelling of haemocytes and subsequent confocal visualisation and analysis were carried out as described in section 2.2.6.

3.2.5 Quantitative analysis of haemocyte phenotypes

Haemocyte monolayers were exposed to 0.75 mg ml^{-1} of W14 particulate preparations of *P. luminescens*W14 wildtype and *tc*- mutant strains. Seven monolayers were prepared from each insect for each of the seven treatments to

enable direct comparison between the set of monolayers, three insects were used per treatment. Confocal microscopic analyses were carried out on random views of each monolayer and at least 100 cells per cell type (granulocyte or plasmatocyte) were examined categorised by their actin phenotype (granulocytes; polarised, diffused or punctate with actin foci and plasmatocytes; diffused, stress fibres, actin cables, punctate with actin foci or blebbed membrane). The percentage of 'control' phenotype of granulocytes with polarised actin and plasmatocytes with diffused actin were calculated per insect per treatment and the mean and standard deviation graphed.

3.2.6 *In vitro* phagocytosis assays of *E. coli* by haemocyte

Haemocyte monolayers were prepared as described in section 2.2.8 were incubated with supernatant or cell lysate of *E. coli* ± [tcb^{W14}] or W14 wildtype or *tc*- mutant supernatants at 30°C for 4 h. Phagocytosis assays were carried out as described in section 2.2.8.

3.2.7 Western analysis with anti-Tcb monoclonal antibody

E. coli ± [tcb^{W14}] cultures were aerated at 37°C with samples extracted at 12, 24 and 48 h post inoculation. The samples were adjusted in volume to contain similar cell counts based on OD_{600} and sonicated as described in section 5.2.6. The bacterial cell lysate or supernatant samples were mixed with 2x loading buffer at 1:1 ratio and heated at 95°C for 5 min then chilled on ice for 5 min. Equal amounts were loaded into 8% SDS-PAGE gels along with 20 µl of all samples along with pre-stained broad-range protein markers (7 - 175 kDa; NEB) (Sambrook *et al.*, 1989), electrophoresed at 120 mV. One gel was stained with Coomassie Brilliant Blue (Sigma) and the other was blotted onto PVDF membrane for Western analysis.

The blots were used for Western analysis against a mouse monoclonal anti-Tcb antibody. Membranes were blocked in 0.2% Tween-20 (vol/vol) and 5% powdered milk (wt/vol) in PBS for a minimum of 1 h and then immuno-blotted with primary

antibodies (1 in 50) for 2 h. Detection was carried out using alkaline-phosphatase conjugated anti-rabbit or anti-mouse (Sigma) at 1 in 10,000 dilution for 1 h. Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) liquid substrate system (Sigma).

3.3 Results

3.3.1 Oral toxicity of W14 wildtype and *tc* knockout mutants' supernatants

Oral toxicity is a well-characterised phenotype for Toxin complex (Tc) toxins TcA and TcD of *P. luminescens* W14. To confirm this phenotype for the *tc*- mutant strains used for experiments, oral bioassays with *P. luminescens* W14 wildtype and *tc*-knockout mutant strains were carried our for oral toxicity against *M. sexta* neonates. The first instar larvae were fed on discs of artificial diet laced with supernatants were incubated at 25°C over 72 h, the larval weight monitored (Fig. 3.1a) and larval viability scored (Fig. 3.1b). Control larvae were fed on artificial diet discs laced with *P. temperata* K122 supernatant under the same conditions. Data show *P. luminescens* W14 wildtype, *tcb* and *tcc* mutants to inhibit growth to less than 10% of control, while *tca* knockout (*tca*-) mutant restricts growth to 23 ± 1% relative to control. Further, *tcd* knockout (*tcd*-) and *tca*+*tcd* knockout (*tca-/ tcd-)* mutants have no effect on larval weight gain relative to control (Fig. 3.1a). Wildtype *P. luminescens* W14 supernatant treatment caused 100% larval mortality after 72 h while *tcb*- and *tcc*- mutants caused reduced percentage larval mortality 66.3 ± 19.3 % and 55 ± 11 %, respectively. The *tca*- mutant supernatant further reduced the percentage larval mortality to 11 ± 11 % and no death was observed in larvae fed on either *tcd*- or *tca-/ tcd*- mutant supernatants (Fig. 3.1b).

In summary, the oral toxicity phenotypes of mortality and inhibited weight are due mainly to *tcd*, but both weight gain and mortality are also influenced by *tca* since the respective mutants for both genes inhibit these phenotypes. The other *tc* loci appear to contribute to a smaller extent towards mortality, but did not significantly affect weight gain.

3.3.2 *In vitro* effects of W14 tc toxins of haemocyte mortality

Figure 3.1a illustrates the traits of larval growth inhibition and larval death to be strongly associated principally Tca and Tcd while Tcb and Tcc have less strong

effects, in agreement with the previous findings by Bowen and co-workers (1998). To determine if these toxins are also active against haemocytes, haemocyte monolayers were exposed to supernatants of wildtype *P. temperata* K122, which is non-toxic with 4 h incubation but causes ~60% death after 24 h exposure (Fig 3.2). Haemocyte monolayers were also exposed to recombinant K122 expressing tca^{W14}, tcd^{W14} or $tcd{+}tccC^{W14}$ (Fig. 3.2a). The viability of the treated haemocytes was determined using trypan blue staining to distinguish the viable and dead cells (Fig. 3.2b). The data show the recombinant expression of either Tca or Tcd in K122 disrupts the normally cytotoxic effects of this bacteria strain on haemocytes after 24 h treatment *in vitro*. However, the 24 h treatment cytotoxicity is restored when $TccC^{W14}$ is co-expressed alongside Tcd (Fig. 3.2b).

Cell-free supernatants of wildtype and *tc-* mutants of *P. luminescens* W14 were also assessed for toxicity against haemocytes using the same *in vitro* assay (Fig. 3.3). Intriguingly, the *tca-* mutant induced similar percentage of haemocyte mortality to that of wildtype W14 for both 4 h and 24 h treatments, reflecting the irrelevance of this gene to the cytotoxicity of supernatant shown in Figure 3.2. Additionally, the data also reveal the *tcc-* mutant to induce much-reduced haemocyte mortality (24 ± 2 %) at 24 h suggesting this gene product must also play an important role in haemocyte toxicity (Figure 3.2). Meanwhile, *tcb-*, *tcd-* and *tca-/tcd-* mutant supernatant treatments all caused reduced mortality to 10% or less at 4 h, and approximately 50% at 24 h, suggesting these genes to have significant and relatively minor contributions to whole supernatant cytotoxicity.

3.3.3 *In vitro* effects of W14 Tc toxins of haemocyte actin morphology

Haemocyte actin cytoskeleton rearrangement is another supernatant-induced phenotype of *P. luminescens* W14 as shown in figures 2.5 and 2.6. To determine if the Tc toxins are accountable, Tc-treated haemocyte was examined for -associated Tc- associated actin phenotypes. Haemocyte monolayers were exposed to cell-free supernatants (Fig. 3.4) and particulate preparations (Fig. 3.5) of *P. luminescens* W14 wildtype and *tc-* mutant strains for 4 h at 30°C. The monolayers were stained with TRITC-phalloidin to visualise actin distribution within the treated haemocytes.

PP3 media-only control treated haemocytes display the typical *in vitro* actin phenotypes previously observed (as described in section 2.3.5); granulocytes with polarised actin and multi-morphic plasmatocytes (spindle-shaped, round or irregular) with diffused actin throughout the cell body (Fig. 3.4 and 3.5 panels a, g and m). Confocal analysis shows that *P. luminescens* W14 *tca-* and *tcc-* supernatants disrupt the actin polarity and induced microspikes in granulocytes as wildtype, however, supernatant for the *tcd-* mutant disrupts only some of the granulocytes (Fig. 3.4 panel f) while *tcb-* supernatant treatment leaves the granulocyte actin phenotype unaffected (Fig. 3.4 panel d) similar to that seem in control (Fig. 3.4 panel a). All supernatant treatments induced stress fibres in plasmatocytes with varying degrees of severity, a phenotype which is distinct from that seen in PP3 control treated plasmatocytes with diffused actin (Fig. 3.4 panels g - l). Also noted is the condensed actin foci phenotype, which was induced by wildtype *P. luminescens* W14 treatment only. Haemocytes with actin-rich protrusions were observed in all treatments except PP3 media only control (Fig. 3.4 panels m - r).

Haemocyte actin phenotypes were more wide-ranging when treated with particle preparations enriched in high molecular contents including Tc toxins (Fig. 3.5). In agreement to the results of treatment with supernatants, granulocyte actin polarity was disrupted by particle preparations from all mutant strains with the exception of the *tcb-* mutant, leaving all granulocytes with the control phenotype with polarised actin (Fig. 3.5 panels a – f). The *tcc-* mutant particulate treatment induced microspikes in some of the granulocytes on the monolayer (Fig. 3.5e). The confocal images also revealed that *tca-* mutant particulate treatment induced similar plasmatocyte actin phenotypes to that by wildtype W14 (Fig. 3.5 panels i and o). In contrast, the *tcb-* mutant particulate treatment induced control-like plasmatocyte actin phenotype of diffused actin (Fig. 3.5 panels j and p). Lastly, *tcc-* and *tcd-* mutant particle treatments induced some wildtype-like plasmatocyte actin phenotypes while many plasmatocytes are left with diffused actin (Fig. 3.5 panels k, l, q and r).

3.3.4 Quantitative analysis of W14 Tc toxins treated haemocyte actin phenotypes

Figures 3.4 and 3.5 illustrate changes in haemocyte actin phenotypes when treated with particulate preparations of *P. luminescens* W14 wildtype and *tc*- mutant strains. In order to correlate any specific changes to particular *tc* loci, a survey was conducted to determine the frequency of granulocytes and plasmatocytes with control-like or *P. luminescens* W14-supernatant induced-like actin phenotypes (Fig. 3.6 and 3.7).

Figure 3.6a shows the three observed granulocyte actin phenotypes, including a novel granulocyte actin phenotype displaying condensed actin foci throughout the granulocyte cell body not observed previously along side polarised and microspike phenotypes. Figure 3.6b shows the percentage of granulocytes with polarised actin relative to the total granulocyte population examined. The histogram shows *P. luminescens* W14 wildtype, *tca*- and *tca-/tcd*- mutant particulate treatments cause microspikes in all granulocyte examined. While PP3 media only control treated monolayer show 100% granulocytes with polarised actin, the *tcb*- and *tcc*- mutant treatments 73.2 ± 2.2 % and 8.9 ± 2.3 % of granulocytes, respectively, with control-like granulocytes.

The same study was carried out for plasmatocytes with five actin phenotype categories: diffused actin, stress fibres, actin cables, condensed actin foci and blebbed membranes (Fig. 3.7a). The histogram displays the percentage of plasmatocytes observed with diffused actin phenotype relative to the total plasmatocyte population examined (Fig. 3.7b). The *tca*- particulate treatment left 9.1 ± 4.5 % plasmatocytes with diffused actin, which is similar to that of wildtype treatment at 7.5 ± 5 %. Treatment with *tcd*- mutant particulate preparations yielded a higher percentage of diffused plasmatocytes at 79.1 ± 5.6 %, which is close to the PP3 media only control at 97.2 ± 1.2 %. Treatments with *tcb*-, *tcc*- and *tca-/tcd*- have resulted in around 50% of plasmatocytes with diffused actin.

3.3.5 *In vitro* effects of W14 supernatants on haemocytes phagocytic competence

Since phagocytosis is dependent on actin dynamics, these subtle changes in haemocyte actin phenotypes observed between the wildtype and the *tc*- mutant treatments suggest that the Tc toxins may inhibit phagocytosis. To assess the phagocytic ability of haemocyte treated with *P. luminescens* W14 wildtype and *tc*- mutant strains, haemocyte monolayers were exposed to supernatant of wildtype and *tc*- mutant strains and incubated to GFP-*E. coli* at 25°C for 4 h (Fig 3.8). The solid bars on the scatter graph indicate the mean percentage phagocytes; PP3 control treatment is at 72.3 ± 9.1% and for *P. luminescens* W14 wildtype supernatant treatment at 5.7 ± 1.9 % emphasising the active suppression. All *tc*- mutant treatments reduced the percentage phagocytosis to 21.6 % or less, except *tcb*-, which achieved a slightly higher average at 29.6 ± 34.1 % due to three experiments of nine yielding PP3 control-like levels of phagocytosis.

3.3.6 *In vitro* effects of recombinant Tcb on granulocyte actin morphology and haemocyte phagocytosis

Previous studies revealed the *tcb*- mutant treatment results in diminished frequency of treated haemocytes with *P. luminescens* W14 wildtype-induced actin phenotypes. These findings argue that Tcb is a functional factor in the supernatant of *P. luminescens* W14 with role(s) in haemocyte actin rearrangement. Such effects would aid the pathogen to destroy or evade the immuno-active haemocytes, which is necessary for successful infection.

To assess the effects of Tcb on haemocyte actin phenotypes, haemocyte monolayers were exposed to Tcb expressed in *E. coli* and then the granulocyte actin phenotype and the haemocytes ability to phagocytose were examined. Firstly, cell-free supernatants or cell lysates were prepared from 12, 24 or 48 h old cultures of *E. coli* ± [tcb^{W14}]. Western analysis was performed using a monoclonal anti-Tcb antibody against the supernatant and cell lysate samples to confirm Tcb expression (Fig. 3.9a).

The cell lysate samples give an intense signal at approximately 25 kDa and three weaker signals at molecular mass of 83 kDa or greater, which are absent from the *E. coli* without plasmid control. Only a weak signal at 25 kDa is detectable in the 24 h supernatant sample.

To determine if Tcb^{W14} alone is sufficient to alter granulocyte actin phenotypes, haemocyte monolayers were exposed to the *E. coli* [tcb^{W14}] cell lysate or supernatant samples *in vitro* and the actin phenotype examined (Fig. 3.9b). The confocal images reveal sustained actin polarity in the treated granulocytes indistinguishable from the control granulocyte actin phenotype. *M. sexta* granulocytes have been shown to phagocytose *E. coli in vitro* and *in vivo*, which is inhibited in the presence of wildtype *P. luminescens* W14 supernatant (section 2.3.8) and figure 3.8 shows inconsistent results indicating a possible functional role of Tcb to suppress phagocytosis of *E. coli*. To investigate this, phagocytosis assays were performed exposing haemocyte monolayers to the supernatant or cell lysate fractions of *E. coli* ± [tcb^{W14}] and GFP-*E. coli*. The histogram shows there is no difference between Tcb and control treatments in the percentage phagocytic haemocytes regardless of whether cell lysate or culture supernatants were applied (Fig. 3.9).

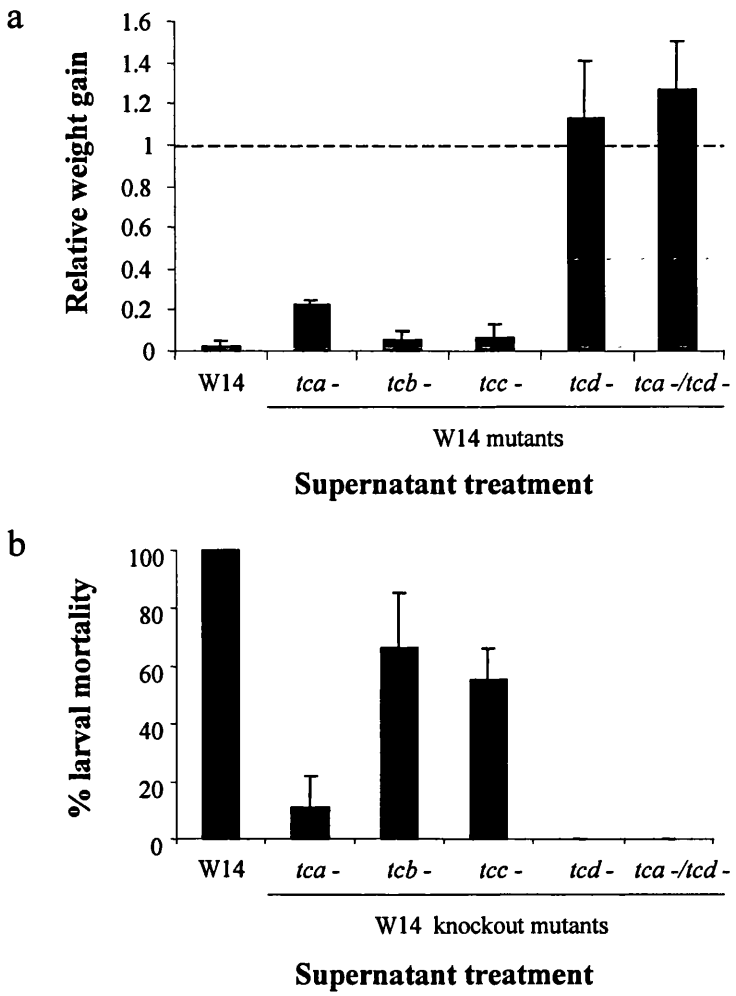


Figure 3.1 Oral bioassays with W14 wildtype and *tc* knockout supernatants treatments. First instar *M. sexta* larvae were fed on discs of artificial wheat germ diet laced with cell-free supernatant of wildtype and *tc* knockout mutant strains of *P. luminescens* W14 incubated at 25°C for 72 h. The histograms above illustrate larval weight gain relative to non-orally toxic K122 supernatant indicated by dotted line (a) and viability (b) of the larvae. Note wildtype, *tcb*- and *tcc*- mutant strains inhibited growth to less than 10% of control weight, *tca*- at 23% relative to control, while *tcd*- and *tcd-/tcd*- mutants with weight gain greater than control (a). Also W14 wildtype supernatant caused 100% mortality, while *tcb*-and *tcc*- mutants resulted more than 50% mortality. However, *tca*-, *tcd*- and *tca-/tcd*- mutants caused reduced mortality at 10% or less (b). Data represent means and standard deviations of three experiments using three larvae per treatment per experiment.

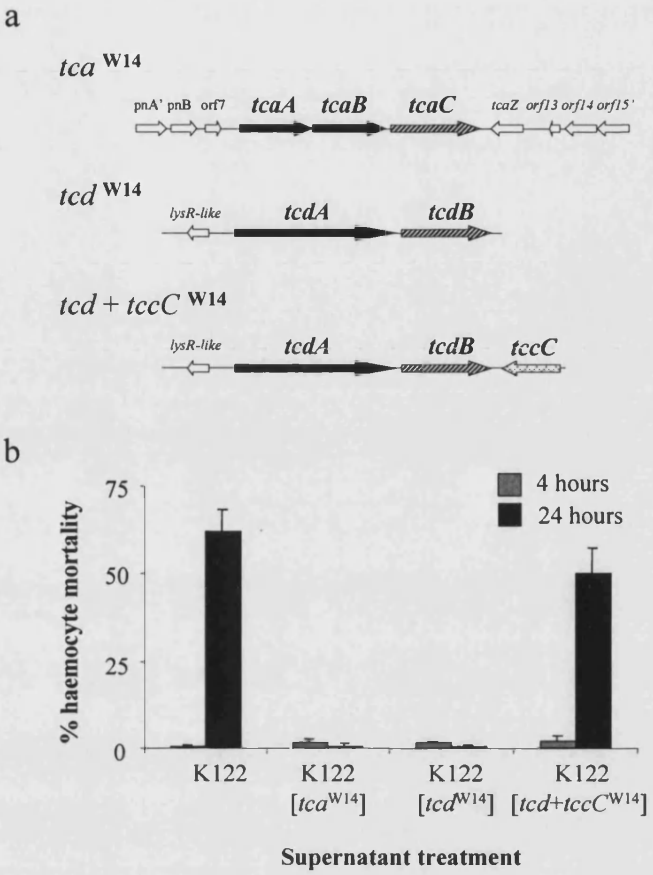


Figure 3.2 Effects of W14 *tca* and *tcd* ± *tccC* on haemocyte mortality *in vitro*. Panel a illustrates the recombinant constructs carrying orally toxic loci *tca* or *tcd*-derived *P. luminescens* W14 fragments expressed in the non-orally toxic K122 (adapted from Waterfield *et al.*, 2001a). The tca^{W14} construct has a Bluescript pBCKS(+) plasmid backbone and the *tcd*-derived constructs have a pBR322 plasmid backbone. Haemocyte monolayers were treated with supernatants of the recombinant K122 clones *in vitro* at 30°C for 4 h or 24 h and the haemocyte viability assessed (Fig. 3.2b). Note the non-orally toxic K122 supernatant cause low haemocyte mortality after 4 h treatment and a higher mortality count of 62.2 ± 6.3% after 24 h treatment. The recombinant expression of Tca or Tcd in K122 diminishes the haemocyte mortality after 24 h treatment, note the expression of TccC with Tcd reverses the diminishing effect. Data represent the means and standard deviation of at least 500 haemocytes counted per monolayer per insect, three insects per treatment.

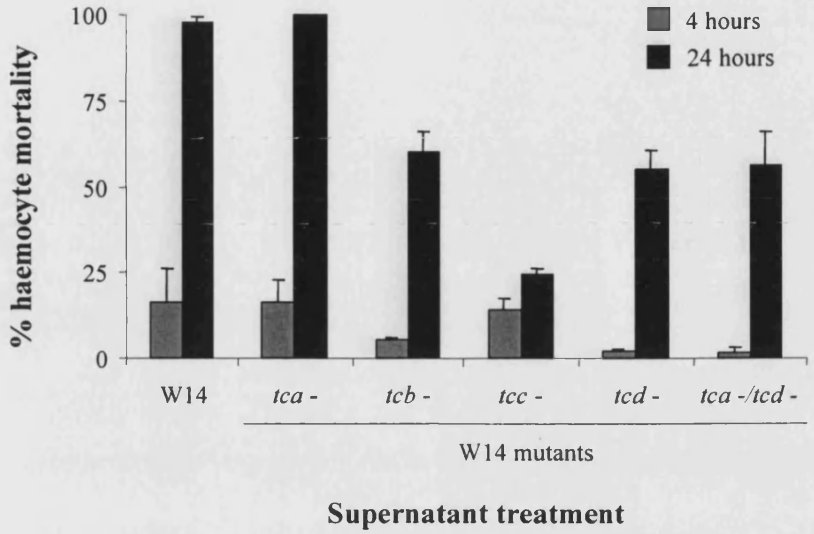


Figure 3.3 Effects of W14 Tc toxins on haemocyte mortality *in vitro*. Haemocyte monolayers were treated with supernatants of *P. luminescens* W14 wildtype and *tc*- mutant strains *in vitro* at 30°C for 4 or 24 h and the haemocyte viability determined. The data show W14 wildtype and *tca*- mutant strain supernatants to cause similar haemocyte mortality rates. Note haemocytes treated with *tcb*-, *tcd*- and *tca-/tcd*- mutant supernatants for 4 h cause reduced mortality at less than 10% while *tcc*- mutant supernatant treatment yielded similar haemocyte mortality to wildtype. Also note after 24 h treatment, all *tc*- mutant supernatants, except *tca*-, cause reduced percentage haemocyte death relative to wildtype. Notably, *tcc*- mutant supernatant treatment caused the lowest haemocyte mortality after 24 h while *tcb*-, *tcd*- and *tca-/tcd*- mutant supernatants cause half the haemocyte mortality of that of wildtype. Data represent the means and standard deviation of at least 500 haemocytes counted per monolayer per insect, three insects per treatment.

Figure 3.4 Effects of W14 Tc toxins on haemocyte actin cytoskeleton *in vitro*. Haemocyte monolayers were treated with supernatants of *P. luminescens* W14 wildtype and *tc*-mutant strains *in vitro* at 30°C for 4 h. The haemocyte actin structures are visualised using fluorescein-conjugated phalloidin. Confocal analysis reveals PP3 media-only control treated haemocytes with typical *in vitro* actin phenotypes (single arrowheads); granulocytes with polarised actin (panel a) and general diffused actin in plasmatocytes (panels g and m). Also shown are the actin phenotypes of *P. luminescens* W14 supernatant treated granulocytes (panels b – f) and plasmatocytes (panels h – r). Note *tcb*- mutant supernatant treated granulocytes retain actin polarity (panels d) resembling PP3 control treated granulocytes (panel a), while *tcd*- mutant supernatant treated monolayers display some polarised granulocytes (single arrowhead in f) and some with microspikes (double arrowhead in b - f). All other *tc*- mutant supernatant treatments yielded actin structure similar to that of W14 wildtype supernatant treatment with microspikes (double arrowhead in b). All *tc*- mutant supernatant treatments yielded similar plasmatocyte actin phenotypes to that of wildtype supernatant treatment, stress fibres (panels h – l) and condensed actin-cables (panels n – r). All images shown in figure 3.4 were taken from monolayers prepared from the same larva in order to enable direct comparison; at least 200 haemocytes were examined per monolayer and three independent experiments were carried out yielding similar results as represented in the figure.

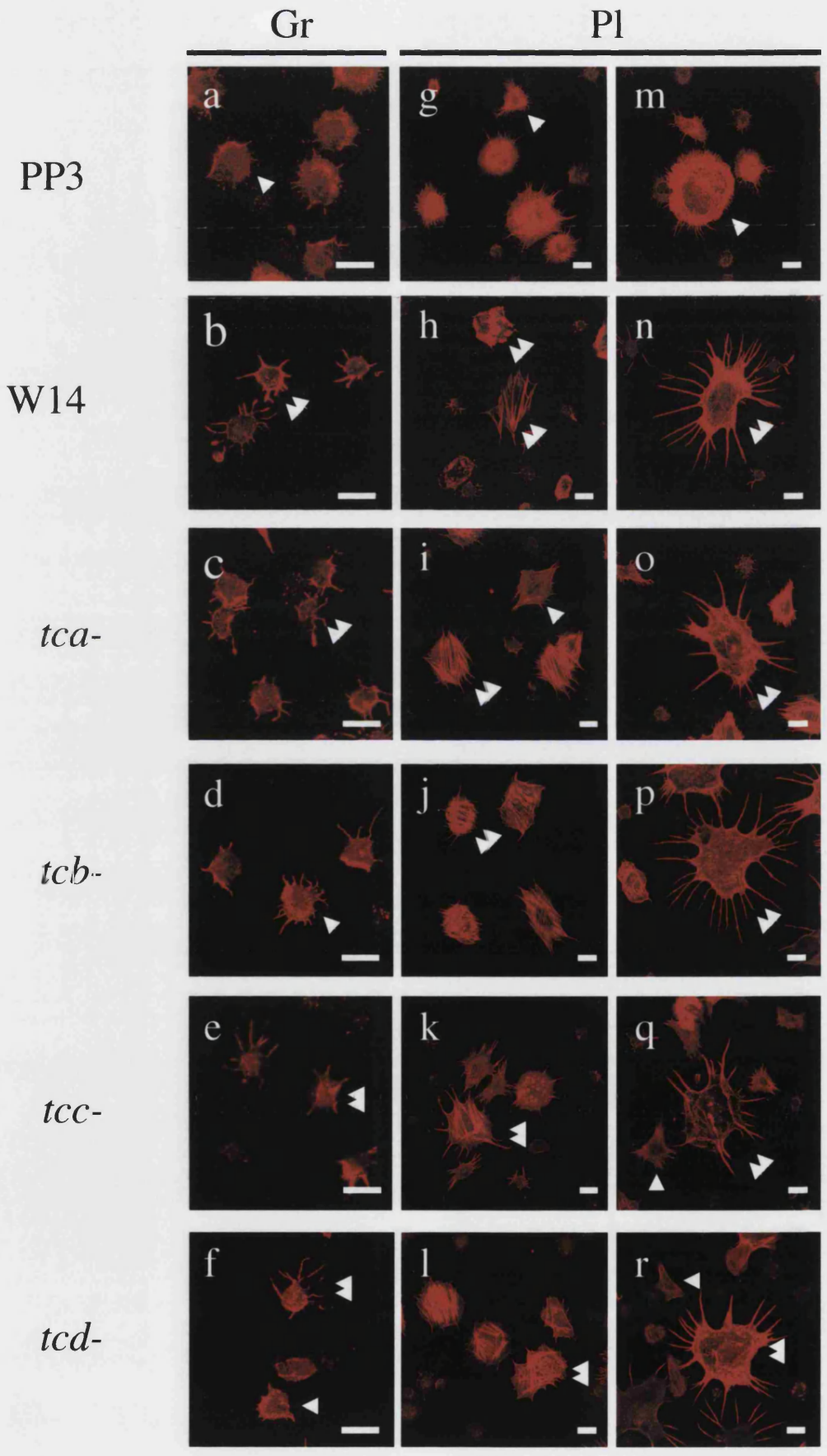

Gr
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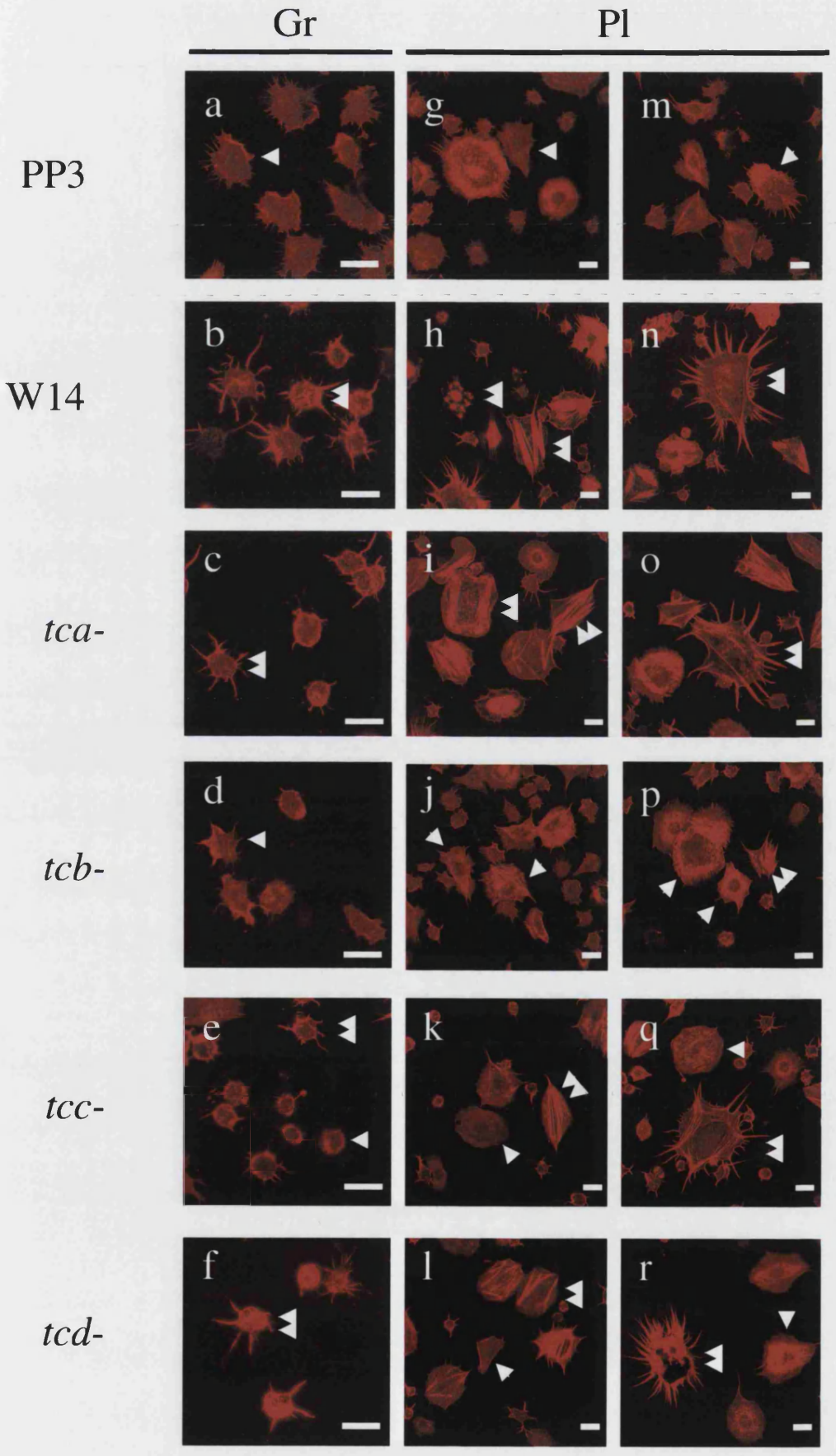
Figure 3.5 Effects of enriched W14 Tc toxins on haemocyte actin cytoskeleton *in vitro*. Haemocyte monolayers were treated with particulate preparations from cell-free supernatant of *P. luminescens* W14 wildtype and *tc-* mutant strains *in vitro* at 30°C for 4 h. Figure 3.5 shows the actin cytoskeleton of granulocytes (panels b – f) and plasmatocytes (panels h – l and n - r) treated with particulate preparations of wildtype or *tc-* mutant. The preparation procedure particulate fraction enriches high molecular contents of the supernatants including the Tc toxins. Haemocytes treated with PP3 control show the typical actin phenotypes (single arrowheads); polarised actin distribution in granulocytes (panel a) and general diffused actin in plasmatocytes (panels g and m). Only *tca-* mutant particulate treatment yielded actin phenotypes similar to that of W14 wildtype supernatant treatment; plasmatocytes displaying stress fibres, condensed actin foci and actin-cables (double arrowheads in panels h, i, n and o). Note *tcb-* mutant particulate treated granulocytes (panel d) retain actin polarity resembling PP3 control treated granulocytes (panel a), while *tcc-* mutant particulate treated monolayers display some polarised granulocytes (single arrowhead in panel e) and some with microspikes (double arrowhead in panel e). All other mutant particulate treatments caused granulocyte actin rearrangements similar to that of wildtype particulate treatment inducing microspikes (double arrowhead in panel b). While *tcb-* mutant treated plasmatocytes have actin phenotypes similar to PP3 control, haemocytes treated with particulate preparations of *tcc-* and *tcd-*, displayed a mixture of control-like (single arrowheads) and wildtype-like (double arrowheads) actin phenotypes (panels k, l, q and r). All images shown in this figure are taken from monolayers prepared from the same larva in order to enable direct comparison; at least 200 haemocytes were examined per monolayer and three independent experiments were carried out yielding similar results as represented in the figure.


Gr
Pl
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tca-
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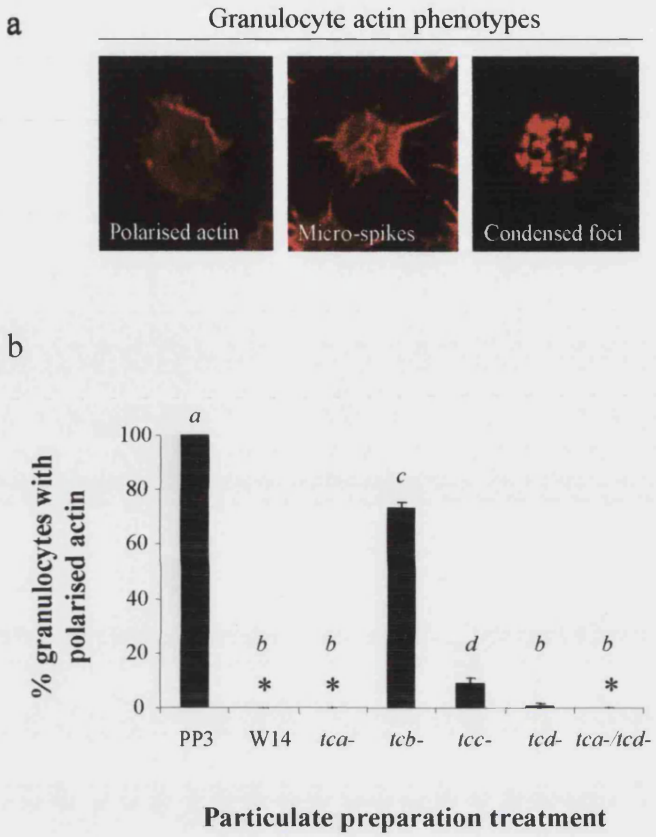


Figure 3.6 Quantitative analysis of granulocyte actin phenotypes treated with Tc toxins enriched particulate preparations from W14. Haemocyte monolayers were treated with particulate preparations from wildtype or *tc*- mutants of *P. luminescens* W14 at 30°C for 4 h. The granulocyte actin structures were examined and the actin phenotypes categorised into one of three granulocyte-actin phenotypes shown in figure 3.6a; polarised actin, micro-spike and condensed foci. Figure 3.6b shows the percentage of granulocytes with polarised actin, PP3 control treatment leaves almost all granulocytes with polarised actin while wildtype W14 disrupts the polarity in all granulocytes. Note that *tca*-, *tcd*- and *tca*-/*tcd*- mutant treatments cause similar loss in granulocyte actin polarity to wildtype. Also note the higher count of polarised granulocytes resulted after *tcb*- and *tcc*- mutant treatments at 73.2 ± 2.2% and 8.9 ± 2.3%, respectively. Data represent mean and standard deviation of at least 100 granulocytes counted per monolayer per insect, three insects per experiment. Asterisk (*) = 0%. Means carrying the same letter are not significantly different at the 5% level (paired *t*-test).

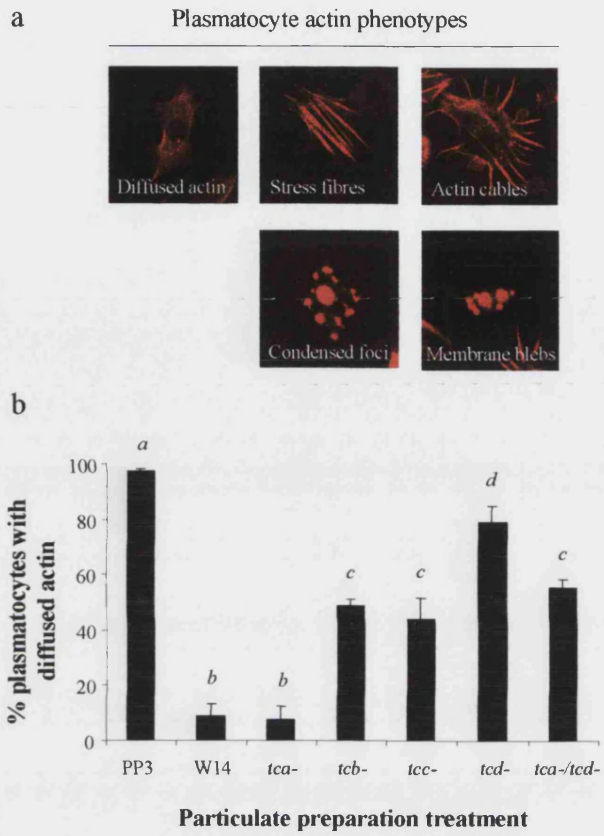


Figure 3.7 Quantitative analysis of plasmatocyte actin phenotypes treated with Tc toxins enriched particulate preparations from W14. Haemocyte monolayers were treated with particulate preparations from wildtype or *tc-* mutants of *P. luminescens* W14 at 30°C for 4 h *in vitro*. The plasmatocyte actin structures were examined and categorised into one of five plasmatocyte-actin phenotypes shown in figure 3.7a; plasmatocytes with diffused actin, stress fibres, actin cables, condensed foci or membrane blebs. Figure 3.7b shows the percentage of plasmatocytes examined with diffused actin, PP3 treatment leave almost all plasmatocytes with diffused actin while wildtype and *tca-* mutant treatments reduced this to 9.1 ± 4.5 % and 7.5 ± 5 %, respectively. Note the high 79.1 ± 5.8 % of *tcd-* mutant treated plasmatocytes retained the diffused actin phenotype, while *tcb-*, *tcc-* and *tca-/tcd-* mutant treatments reduced the diffused actin plasmatocyte count to approximately 50%. Data represent mean and standard deviation at least 100 plasmatocytes counted per monolayer per insect, three insects per experiment. Means carrying the same letter are not significantly different at the 5% level (paired *t*-test).

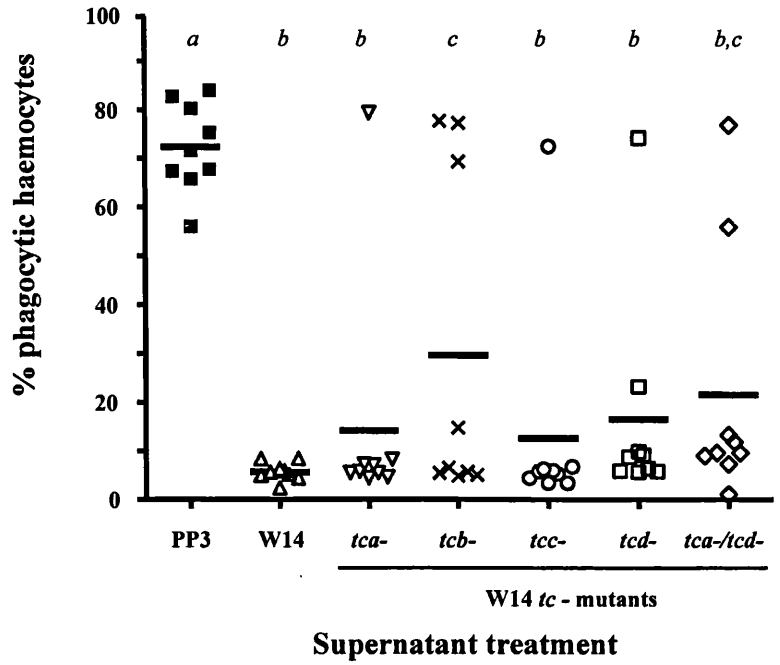
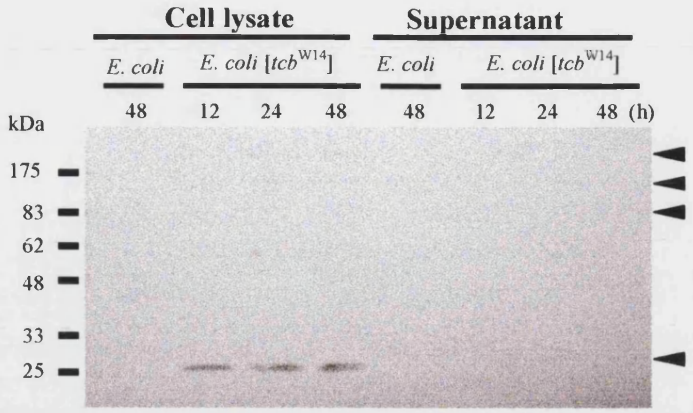
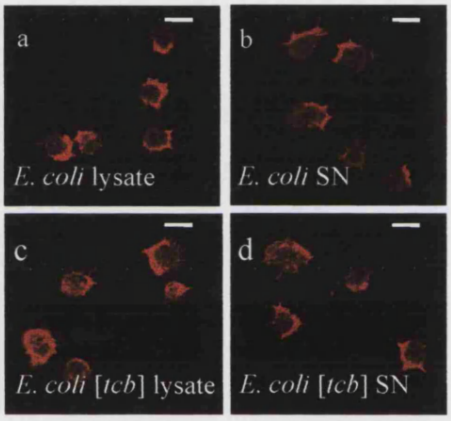


Figure 3.8 Effects of W14 Tc toxins on phagocytosis of *E. coli* by *M. sexta* haemocytes *in vitro*. Haemocyte monolayers were treated with supernatant of W14 wildtype or *tc*- mutant strains and exposed to GPF-*E. coli* given sufficient time and suitable conditions for phagocytosis. The percentage of haemocytes with engulfed GFP-*E. coli* was recorded (Fig. 3.7). Each data point represents the percentage of phagocytic haemocytes of approximately 500 examined per monolayer. Monolayers per experiment were prepared from the same larva to allow direct comparison; three larvae used per experiment; three independent experiments were carried out. Bars represent the mean of nine data points, note the higher average of the *tcb*- mutant supernatant treatment with three data points comparable to that of control. Means carrying the same letter are not significantly different at the 5% level (paired *t*-test).

a - Anti-Tcb western analysis



b – Granulocyte actin phenotype



c – Phagocytosis of *E. coli*

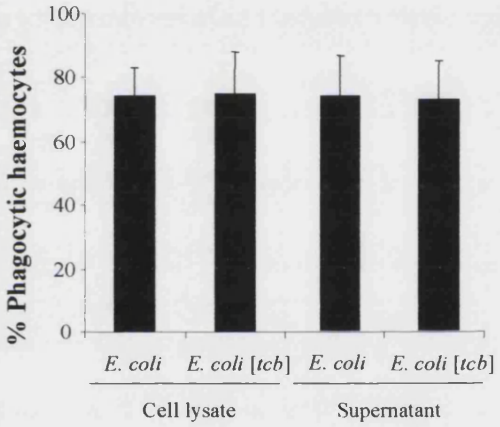


Figure 3.9 Effects of recombinant *tcb*^{W14} on granulocyte actin phenotype and haemocytes phagocytosis of *E. coli in vitro*. Recombinant *E. coli* host was used to express *tcb*^{W14}, an anti-Tcb antibody was used to confirm the presence of Tcb in the cell lysate and supernatant (a). Arrows indicate the positive signals; three at high molecular weight and one at 25kDa. Note the intense signals from the cell lysate samples at 25 kDa and a weaker signal in the supernatant from the 24-h-old sample. Figure 3.8b show representative images of haemocyte monolayers treated with the Tcb expressing samples, note all granulocytes observed have polarised actin distribution (Scale bar = 10 μm). The histogram indicates recombinant *tcb*^{W14} in *E. coli* has no effect on the phagocytic abilities haemocytes *in vitro*. Data represent the means and standard deviations of at least 500 haemocytes counted per monolayer per insect, three insects per treatment.

3.4 Discussion

Four toxin complexes are associated with *P. luminescens* W14 oral toxicity. Original studies showed Tca and Tcd together account for the majority of the supernatant oral toxicity, while Tcb and Tcc were shown to have relatively minor contributions (Bowen and Ensign, 1998). Data suggest complex interactions between the *tc*-encoded products, while the exact protein composition of the active complexes and the component stoichiometry remain obscure.

Several lines of investigation led to proposals that Tca (Marokhazi *et al.*, 2003; Blackburn *et al.*, 1998; Bowen and Ensign, 1998) and Tcd (Marokhazi *et al.*, 2003; Waterfield *et al.*, 2001a; Guo *et al.*, 1999) are associated with oral toxicity. TcaB was shown to be orally toxic against southern corn rootworm (Guo *et al.*, 1999). TccC was proposed to be a toxin or a modulator / activator of a toxin based on the finding that TcdA and TcdB must be co-expressed to confer oral toxicity in *E. coli* (Waterfield *et al.*, 2001a). However, little evidence is available to elucidate the role of the other Tc toxins in *in vivo* infections. *P. luminescens* W14 supernatant-induced effects on haemocytes are described in chapter 2 including induced cell mortality, loss of motility, suppressed phagocytic competency and rearranged actin phenotypes. As Tc toxins are present in the *P. luminescens* W14 supernatant, the present work investigates the possibility that Tc toxins are responsible for these phenotypes.

Firstly, *P. luminescens* W14 wildtype and *tc-* mutant strains cultured from frozen stocks were tested in oral bioassays to check their oral toxicity phenotype. Neonate larvae fed on artificial diet laced with supernatants were monitored for relative weight gain and mortality over 72 h. Larval weight gain data highlight Tca and Tcd as the main factors contributing to inhibition of weight gain (Fig. 3.1a); both these toxin complexes are also implied to contribute strongly towards larval mortality (Fig. 3.1b). These conclusions are in broad agreement with previous studies by Bowen *et al.* (1998), although Tcd appears to be the dominant toxin complex based on data of these studies while Tca appeared to be such in previous studies by Bowen *et al.* (1998). This discrepancy may be resolved by repeating the original experiments

exactly; firstly by testing various dilutions of concentrated supernatant samples, however, since the stoichiometry of the Tcs are unknown, more precise measures are not possible and secondly to extend the experiments over seven days (168 h) rather than three days (72 h). In spite of this, both studies highlight the minor roles of Tcb and Tcc in these phenotypes, which may have alternate targets such as insect host haemocytes.

The Tca protein was shown to cause larval gut damage when consumed orally or by injected directly into larval haemocoel (Blackburn *et al.*, 1998), leaving the host molecular target specificity of Tca open to speculation. Further, Waterfield *et al.* (2001) demonstrated that *P. luminescens* W14 Tcd confers oral toxicity in the non-orally toxic *P. temperata* K122 background while the co-expression of TccC with Tcd is required in a non-pathogenic *E. coli* host, suggesting complex interactions between toxin components. Experiments here were designed to test if the recombinant Tca^{W14} alone, Tcd^{W14} alone or Tcd^{W14} plus $TccC^{W14}$ in the non-toxic *P. temperata* K122 are toxic against haemocytes. The recombinant expression constructs in K122 are illustrated in figure 3.2a (adapted from Waterfield, Dowling *et al.*, 2001).

Wildtype *P. temperata* K122 supernatant was found to be non-toxic against *M. sexta* haemocytes with a 4 h *in vitro* treatment (Fig. 2.3 and section 2.2.3). Figure 3.2b shows little haemocyte death after 4 h incubations with all treatments, although, K122 wildtype supernatant caused 62.1 ± 6.3 % of haemocyte mortality after 24 h. Interestingly, the co-expression of Tca or Tcd diminished this supernatant-induced haemocyte mortality while the co-expression of Tcd with TccC restored mortality to 50.3 ± 6.9 %, close to the level of the non-recombinant K122 control. These cytotoxic traits do not mirror the oral toxicity of these recombinant K122 strains, as Waterfield, Dowling *et al.* (2001) demonstrated either Tca or Tcd in recombinant K122 conferred oral toxicity, therefore the orally toxic Tca and Tcd are unlikely to be active against haemocytes.

A possible explanation for the diminished and reversible cytotoxicity could be disruption of a delicate balance of toxin components present in the supernatant, i.e.

an excess of Tca or Tcd may destabilise the active toxin complexes, which is restored in the presence of an equal molar ratio of TccC. The native protein profiles of wildtype K122 and recombinant K122 particulate preparation profiles are clearly different suggesting interactions between recombinant and endogenous proteins (Waterfield *et al.*, 2001a). It may be interesting to test for changes in supernatant cytotoxicity of *P. luminescens* W14 strains with certain Tcs over-expressed. Despite this unexpected outcome of diminished cytotoxicity, it is encouraging to find that certain W14 Tc components alter the haemocyte toxicity of *P. temperata* K122 bacterial supernatant, suggesting possible roles of Tc toxins in infections *in vivo* common to the two pathogenic strains.

To further investigate the possible role of Tc toxin against haemocytes, *P. luminescens* W14 wildtype and mutant supernatants were overlaid on to haemocyte monolayers to assess haemocyte mortality after 4 and 24 h (Figure 3.3). The data reveal *tcb-* and *tcd-* mutants to cause reduced percentage of haemocyte mortality after 4 h and *tcb-*, *tcc-* and *tcd-* to cause reduced percentage of haemocyte mortality after 24 h, indicative of involvement in cytotoxic effects of wildtype supernatants. Interestingly, *tca-* supernatant treatment caused very similar haemocyte death count to that of wildtype W14 supernatant after 4 and 24 h, implying its irrelevance for anti-haemocytic functions seen *in vitro*. This is consolidated by the similar reduction in haemocyte death count caused by the *tcd-* single knockout supernatant treatment compared to that of the *tca- /tcd-* double knockout mutant.

The results obtained are unexpected since *tcaB* shares 53% identity to *tcbA* and *tcdA* hence one may expect a similar function of the conserved region. Also TcaC bears 48% amino acid sequence homology to *Salmonella* plasmid-borne virulence factor protein SpvB (Waterfield *et al.*, 2001b) shown to enhance bacterial survival in macrophages (Libby *et al.*, 2000) as well as a carboxyl-terminal ADP-ribosyltransferase, both of which appear not to be functional against *M. sexta* haemocytes in these *in vitro* assays.

Intriguingly, the *tcc-* supernatant treatment of 24 h caused the lowest percentage of haemocyte mortality (Fig 3.3), suggesting the possible importance of Tcc in

haemocyte-killing. Additionally, the co-expression of $TccC^{W14}$ and Tcd^{W14} in K122 restored the diminished supernatant cytotoxicity by Tca^{W14} and Tcd^{W14} (Fig. 3.2), again implying that TccC to be involved in toxicity. This should be further investigated since TccC has been shown to have a crucial role in oral toxicity in Tcd-expressing *E. coli* (Waterfield *et al.*, 2001a). I also investigated the effects of Tc toxins on phagocytosis of *E. coli* by haemocytes since *Photorhabdus* clearly survive in the insect; hence it is probable that they evade the cellular defence reactions of the host haemocytes. As the haemocytes must be prevented from phagocytosing invading cells instantly upon contact of the offending bacteria without prolonged incubation of 24 h, experiments were carried out to study the more immediate effects such as the actin structural changes of the haemocytes within a 4 h treatment.

Both supernatant and particulate preparations of *P. luminescens* W14 wildtype and *tc-* mutant were tested against haemocyte monolayers and the haemocyte actin phenotypes examined. Previous analysis revealed several wildtype W14 supernatant-induced actin phenotypes for granulocytes; polarised or with microspikes and plasmatocytes; diffused actin, stress fibres or actin foci (Fig. 2.5 and 2.6). Confocal images of supernatant-treated haemocytes show that wildtype and *tca-* cause the loss of actin polarity in almost all granulocytes and *tcb-* left most granulocytes with control-like actin polarity, hence implying Tca to be irrelevant and Tcb as being important to granulocyte actin polarity disruption. However, *tcd-* treatment caused the loss of actin polarity in some granulocytes, hence may have minor roles. All other *tc-* supernatant treatments induced plasmatocyte actin phenotypes similar to wildtype.

Particulate preparations of *P. luminescens* W14 wildtype and *tc-* mutants were also tested in the same *in vitro* haemocyte mortality assays (Fig. 3.5). These particulate samples contain enriched Tc toxins, which may reveal more information than that of the supernatant samples used in figure 3.4. This was indeed the case as several additional actin phenotypes were seen on the particulate-treated haemocyte monolayers. One additional actin phenotype in granulocytes with condensed actin foci was observed and plasmatocytes with actin cables and blebbed-membranes were uncovered in these particulate-treated monolayers (Fig. 3.5). As with the supernatant

treatment in the previous assay, *tcb*- particulate treatment induced only a few wildtype-like actin phenotypes in granulocytes and plasmatocytes. The *tcc*- particulate treatment induced wildtype-like actin phenotypes in some haemocytes while leaving others control-like. Lastly, *tcd*- particulate treatment induced wildtype-like granulocytes whilst many plasmatocytes maintained diffused actin in the cytoplasm (Fig. 3.5 f, l and r). Collectively, the data suggests Tcb, Tcc and Tcd are potentially anti-haemocytic while Tca appears likely to be irrelevant to these phenotypes.

To quantitatively illustrate these changes in actin phenotypes between the W14-wildtype and *tc*- mutants, the control-like actin phenotypes; polarised granulocytes and diffused plasmatocytes were documented from randomly views of particulate-treated monolayers (Fig. 3.6 and 3.7 respectively). Percentage of granulocytes with polarised actin after 4 h particulate treatments is shown in figure 3.6b along with images of the other granulocyte actin phenotypes (Fig. 3.6a). The histogram reveals *tca*- and *tcd*- to cause actin phenotype changes in almost all the granulocytes examined as with wildtype, while *tcb*- and *tcc*- leave some granulocytes with polarised actin, supporting the possibility of Tcb and Tcc to have anti-haemocytic roles. Similar analysis was carried out for diffused plasmatocytes and results displayed in figure 3.7. Data show all treatments, except for that of *tca*-, yielded higher percentages of plasmatocytes with diffused actin phenotype relative to wildtype, confirming Tca to be unnecessary to the wildtype anti-haemocytic phenotypes while all other *tc*s have potential to contribute.

To find out if Tc toxins affect the phagocytic competence of haemocytes *in vitro* previously observed with wildtype *P. luminescens* W14 supernatant (Fig. 2.10), phagocytosis assays were performed using supernatant of wildtype and *tc*- mutant strains (Fig. 3.8). The results obtained show that *tcb*- and *tca-/tcd*-supernatants show diminished anti-phagocytic effects. Phagocytosis was apparently normal in more than one of nine experiments carried out, showing control-like percentages of phagocytic haemocytes. However, such inconsistencies may result from the failure to grow *P. luminescens* W14 cultures consistently to ensure toxin synthesis and secretion due to incomplete understanding of conditions required. Also noted is that

Tcs are present in wildtype stationary phase supernatant only and mitomycin-C stress was necessary to induce oral toxicity in *E. coli* expressing TcdAB with TccC (Waterfield *et al.*, 2001a). Furthermore, the mode of Tc secretion is as yet unknown and it has been previously speculated that these toxins are released by cell lysis mediated by phage-like genes found in *tc* containing pathogenicity islands (ffrench-Constant *et al.*, 2003; Waterfield *et al.*, 2001b). Nevertheless, the *tcb*- treatment gave rise to control-like results most frequently in three of nine experiments performed, hence *tcb* was chosen to be expressed in recombinant *E. coli* for further investigation.

Previous heterologously expressed Tcs in *E. coli* were shown to lack post-translational processing and secretion (Bowen *et al.*, 1998), hence Western-blot analysis was performed to find the sub-cellular *E. coli*-fraction(s) to contain the recombinant Tcb. The monoclonal anti-Tcb antibody reacted positively against proteins of various sizes in the whole lysate fraction (Fig. 3.9a) and relatively faint against the supernatant fractions, confirming Tcb is restricted to the *E.coli* cytoplasm without secretion. However, haemocytes exposed to the Tcb-containing supernatant and particulate fractions did not exhibit any reduction in either viability or phagocytic competency (Fig. 3.9b and c), suggesting that while Tcb may be a crucial cytotoxic component in *P. luminescens* W14 supernatant, it alone is not sufficient to confer cytotoxicity when expressed alone in *E. coli*. Many possible scenarios can explain this; simplest being that Tcb is an inactive toxin which requires processing for cytotoxic activity, or Tcb could be an accessory protein such as the activator of modulator of toxins, chaperone or secretion apparatus of active toxins etc. all of which could lead to loss or diminished cytotoxicity in supernatant or particulate samples in its absence.

These experiments have highlighted several toxin complexes with potential anti-haemocytic roles, namely Tcb, Tcc and Tcd where W14-induced phenotypes are diminished or abolished in its absence in knockout mutants. Daborn *et al.* (2001) demonstrated that both Tca and Tcd are expressed *in vivo* in a *M. sexta* infection where the expression of Tcd occurred before and Tca after death of insect host using RT-PCR and Western-blot analyses, while Silva *et al.* (2002) confirmed the

expression of Tca *in* vivo. Gold-particle labelling of anti-Tca antibody in TEM studies revealed that Tca is associated with bacterial cell membranes.

Several other techniques are currently in use in our laboratory to better our understanding of the biological functions and mode of action of the Tc toxins. Mammalian culture cells are currently in use to characterise Tc-induced cytotoxicity by exposure to toxin preparations to understand the mode of action, or by expression of certain gene fragments to precisely identify functional regions. Yeast-two-hybridisation using Tc fragments as bait may reveal molecular targets of the toxins while sequence homology mining and studies into evolutionary relationships of *tc*-carrying bacteria could also shed light of the biological relevance in bacterial virulence.

Homologues of *tc* loci have already been found in several insect-associated bacteria *Serratia entomophila* and *Yersinia pestis* being insect and human pathogens, respectively (Waterfield *et al.*, 2001b). The three gene products identified to cause disease in *S. entomophila* show similarity to components of all four *P. luminescens* toxin complexes (Hurst *et al.*, 2000). Further, *S. entomophila* occupies its host's (*Costelytra zealandica*) foregut and is not exposed to the insect haemocoel until after insect death (Jackson *et al.*, 2001), suggesting anti-haemocytic effects are not necessary for infection. However, Waterfield *et al.* (2001a) highlight the modular nature of these toxins, pointing out that negative data using any particular model host / target cells should not lead to definite conclusions of lack of toxicity.

Further, the present investigations are based on toxicity against *M. sexta* haemocytes which may restrict the search of toxins with targets from alternate host hence interpretation of data must be cautious. For examples, while *P. luminescens* W14 Tcb appears to have little effect in oral toxicity against *M. sexta* (Bowen *et al.*, 1998), Guo *et al.* (1999) revealed TcbA oral toxicity against Southern Corn Rootworm. Furthermore, studies of gene loci in isolation may also restrict detection of toxicity if toxins work in synergy, for example the presence of proteases is shown

to process TcbA to yield proteolytic products increasing oral toxicity against the southern corn rootworm (Liu *et al.*, 2003).

This chapter shows the use of *in vitro* assays with *tc* knockout mutants of *P. luminescens* W14 and recombinant expression of candidate Tc toxins in non-toxic host background can aid in determining the role of potential toxins and their relevance in *in vivo* infection system. Here, Tcb was identified to have anti-haemocytic roles from *in vitro* studies using knockout strains, however, further assays using Tcb expressed recombinant *E. coli* did not support the hypothesis. The understanding of Tcs characteristics and modes of action, as with all novel toxins, could eventually lead to the development of novel applied uses such as that of insecticidal transgenes.

Chapter 4

Effects of Mcf on *M. sexta* haemocytes

Some data in this chapter was published in Daborn, P.J., Waterfield, N., Silva, C.P., Au, C.P., Sharma, S. and Ffrench-Constant, R.H. (2002) A single *Photorhabdus* gene, makes caterpillars floppy (mcf), allows *Escherichia coli* to persist within and kill insects. *Proc Natl Acad Sci U S A.* **99**: 10742-10747.

4.1 Introduction

The ffrench-Constant *et al.* (2002) sample sequence analysis of the entomopathogenic *P. luminescens* subsp. *akhurstii* strain W14 revealed numerous putative toxins in its genome but until recently, the toxin complexes (Tcs) were the only known toxins with documented pathogenic effects on model insect hosts (chapter 3). To date, little is known concerning the role of specific toxins in insect host death.

Daborn *et al.* (2002) discovered the *mcf* gene (*makes caterpillars floppy*) by screening a *P. luminescens* W14 genomic cosmid library for lethality towards *M. sexta* larvae by injection. A 33 kb cosmid was originally isolated and subsequent random transposon mutagenesis identified a 8kb ORF responsible for the phenotype (Daborn *et al.*, 2002). Caterpillars injected with bacteria expressing *mcf*, or the Mcf protein itself, display a rapid loss of body turgor and die within 24 h post-injection, hence the 'floppy' phenotype (Daborn *et al.*, 2002). This phenotype is similar to that due to wildtype *P. luminescens* W14 infection (Daborn *et al.*, 2002). Since *M. sexta* larvae have a robust immune system and can clear non-pathogenic bacteria such as *E. coli* effectively (Dunn and Drake, 1983; Horohov and Dunn, 1983; Horohov and Dunn, 1982), the presence of Mcf must therefore enable the Mcf-expressing *E. coli* to circumvent the insect immune system and persist to allow the eventual insect host death.

The *mcf* ORF nucleotide sequence shows no overall homology to known genes, however, parts of the predicted amino acid sequence show limited homology to functional domains of three known proteins (Daborn *et al.*, 2002). There is an amino-terminus Bcl-homology 3-like (BH3-like) domain; all proteins carrying only a BH3 domain are pro-apoptotic (Budd, 2001). There is a central hydrophobic region of the Mcf protein that has homology to the translocation domain of *Clostridium difficile* toxin B (Hofmann *et al.*, 1997). Also present in Mcf is a carboxyl-terminus showing similarity to a RTX-like toxin from *A. pleuropneumoniae* (Schaller *et al.*, 1999). These homologous domains are suggestive of possible toxic properties in Mcf. Additionally, analysis of sequence surrounding *mcf* revealed its position alongside loci with homology to the *Legionella pneumophila dotA* and Enterobacteriaceae haemolysins *hecA/hecB* forming a likely pathogenicity island consisting of putative haemocytic toxins (Daborn *et al.*, 2002; Waterfield *et al.*, 2002b).

Daborn *et al.* (2002) reported apoptotic death of midgut epithelial cells in *M. sexta* larvae injected with Mcf-expressing *E. coli*. Dowling *et al.* (2004) presented morphological and biochemical evidence that Mcf induced apoptosis against mammalian tissue culture cells COS-7, NIH 3T3 and HeLa cells. The BH3 domain is a candidate functional domain responsible for inducing apoptosis. The expression of N-terminal half of Mcf containing the BH3 domain and excluding other potential functional domains was sufficient to induce apoptosis (Dowling *et al.*, 2004). This observation suggests a functional BH3 domain to have pro-apoptotic effects, previously known only in plants and mammals (Budd, 2001). The expression of other Mcf domains in cultured cell-lines is currently underway to precisely locate the functional domains (Dowling, personal communication).

The presence of a close *mcf* homologue in *P. luminescens* W14 was subsequently uncovered and termed *mcf2* and *mcf* was renamed *mcf1* (Waterfield *et al.*, 2003). Injection of either Mcf1 or Mcf2 protein into *G. mellonella* larvae caused larval death at similar rates (Waterfield *et al.*, 2003). Interestingly, although the predicted amino acid sequence of Mcf2 shares homology to the central and carboxy-terminal domains to Mcf1, it differs from Mcf1 at the amino-terminus; Mcf2 carries a domain homologous to HrmA-like type-three effector of *Pseudomonas syringae* pv. *tabaci*

instead of a BH3 domain in Mcf1 (Waterfield *et al.*, 2003). Mcf1 and Mcf2 are suggested to be part of a toxin family with conserved central *C. difficile* toxin B-like translocation domain and variable amino-terminal domain (Waterfield *et al.*, 2003). This chapter investigates the effects of Mcf1 on haemocytes using *in vitro* assays and *in vivo* infections, with the aim of assessing its role in the virulence of *Photorhabdus* strains W14 and *P. temperata* K122.

4.2 Material and methods

4.2.1 D.I.C. microscopy and movies of Mcf treated haemocyte monolayers

Haemocyte monolayers were prepared as described in section 2.2.3. Shallow wells were created using silicon multi-well casts (Molecular Probes) with well moulds 9 mm in diameter and 1.5 mm in depth adhered to standard glass 76 x 26 mm microcopy slides. The wells were filled with approximately 27 µl of ~1 mg/ml cytosolic fraction of either *E. coli- pUC18* or *pUC18-mcf* and haemocyte monolayers inverted onto the wells using silicon gel to form a water tight seal. For differential interference contrast (D.I.C.) microscopy imaging, a X40 Fluor water immersion lens was used on an upright Nikon E1000 microscope. Images were collected at 3 min intervals for up to12 h with a Hamamatsu C4880–07 camera controlled by using METAMORPH software (Universal Imaging, Media, PA). The movies were compiled using Quicktime software (Apple, US).

4.2.2 Mcf and cytochalasin D treated haemocyte monolayers

Haemocyte monolayers were prepared as described in section 2.2.3 and placed in wells containing 360µl of GIM of 24-well plates (Nunc). 10% (40 µl) treatments were added and incubated at 30°C for 2 h; cytosolic fraction of either *E. coli pUC18* or *pUC18-mcf1* (~1 mg/ml^{-1} in PBS) or cytochalasin D (used at ~20 µM in DMSO, Sigma). The monolayers were washed three times with GIM and placed into GIM filled wells for storage before staining with phalloidin-TRITC as described in section 2.2.6.

4.2.3 Cytosol preparation of *E. coli* expressing various *mcf*-BH3 mutants and haemocyte monolayer treatment

E. coli expressing various *mcf*-BH3 mutants were grown in 500 ml LB supplemented with 100 $\mu g/ml^{-1}$ ampicillin, aerated at 37°C overnight. The cultures were centrifuged at 5.9 k RCF for 15 min, the supernatants were discarded and the cells washed with 200 ml of ice-cold PBS, all steps were done at 4°C on ice. The cell pellets were carefully resuspended in 20 ml of PBS each and sonicated on ice (amplitude 90% with cycle of 0.3 s on and 1.0 s off) for 10 s three times with 1 min rest between. The bacterial lysates were centrifuged at 5.9 k RCF at 4°C for 15 min to remove unlysed cell and crude membrane debris. The supernatants were ultracentrifuged at 200 k RCF for 2 h at 4°C separating bacterial membrane fraction from soluble cytosol. The pellet, which contained the host cell membrane fraction, was resuspended in 5 ml of PBS. The supernatants contained the cytosol fractions were aliquoted and stored at -20°C until use.

Haemocyte monolayers were prepared as described in section 2.2.3 and placed into 360 µl of GIM in wells of 24-multiwell plates (Nunc). 40 µl of ~1mg/ml samples were added to monolayers in individual wells and incubated for 10 h at 30°C. To prevent the loss of haemocytes in washes, the monolayers were viewed under an inverted light microscope, a minimum of 500 cells from random views were counted for blebbing membrane.

4.2.4 Western analysis for Mcf expression *in vitro* and *in vivo*

A W14 culture grown in 200 ml of 2% PP3, aerated at 30°C with samples extracted at designated optical density readings (OD_{600}). The samples were adjusted in volume to contain similar cell counts based on OD_{600} for equal loading into SDS-PAGE gels. The bacterial cells were mixed with 2x loading buffer at 1:1 ratio and heated at 95°C for 5 min then chilled on ice. 20 µl of all samples along with pre-stained broad-range protein markers (7 - 175 kDa; NEB) were loaded into two identical 8% SDS-PAGE gel (Sambrook *et al.*, 1989), electrophoresed at 120 mV.

One gel was stained with Coomassie Brilliant Blue (Sigma) and the other was blotted onto PDVF membrane for Western analysis.

First-day fifth instar larvae were injected with $1x10^5$ cells of *E. coli* or W14 and bled at 24 and 48 h post-injection. The samples were centrifuged at 16.5 k RCF for 10 min at 4°C, the pellet resuspended in 1x loading buffer, heated at 95°C for 5 min and kept on ice immediately. The samples were electrophoresed as above, again with one of two identical gels Coomassie stained and one for Western analysis.

The blots were used for Western analysis using two antibodies; an anti-serum raised in rabbit against the W14 Mcf1 domain homologous to *Clostridium* toxin B (shown to be reactive against denatured full-length Mcf in Western analysis) and a mouse monoclonal against the same domain (shown to be reactive against recombinant Mcf (W14) expressed in *E. coli* as well as several host proteins). Membranes were blocked in 0.2% Tween-20 (vol/vol) and 5% powered milk (wt/vol) in PBS for a minimum of 1 h and then immuno-blotted with primary antibodies (1 in 50) for 2 h. Detection was carried out using alkaline-phosphatase conjugated anti-rabbit or anti-mouse (Sigma) at 1 in 10,000 dilution for 1 h. Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) liquid substrate system (Sigma).

4.2.5 Determination of LT_{50} of *Photorhabdus* K122 in *M. sexta*

Photorhabdus K122 wildtype ($K122^{WT}$) and *mcf*-knockout mutant ($K122^{MCF-}$) were grown to mid-exponential growth phase in LB aerated at 30°C. The medium was supplemented with 25 $\mu g/ml^{-1}$ rifampicin (Sigma) for wildtype plus 30 $\mu g.ml^{-1}$ kanamycin (Sigma) for the knockout mutant. Newly moulted first-day fifth *M. sexta* larvae were chilled on ice for 10 min and surface sterilised with ethanol then injected with 100 washed cells directly into the haemocoel using a 100 µl Hamilton syringe with a 30-gauge needle. The larvae were incubated at 25°C and scored for death regularly until the death of all the larvae seven days post-injection.

4.2.6 Comparison of *in vitro* growth between *Photorhabdus* K122 wildtype and *mcf*-knockout mutant

Photorhabdus K122 wildtype ($K122^{WT}$) and *mcf*-knockout mutant ($K122^{MCF-}$) grown in LB supplemented with 25 μg/ml rifampicin (Sigma) for wildtype, plus 30 μg/ml kanamycin (Sigma) for knockout mutant aerated at 30°C. The optical density (OD_{600}) of the cultures was monitored over 24 h and serial dilutions of all samples extracted were plated on LB agar plates with appropriate antibiotics to determine the number of recoverable colony forming units (cfu).

4.2.7 Growth of *Photorhabdus* K122 wildtype and *mcf*-knockout mutant in *M. sexta* and the hosts' total haemocyte count

M. sexta larvae were injected with *Photorhabdus* K122 wildtype ($K122^{WT}$) or *mcf*-knockout mutant ($K122^{MCF-}$) as described in section 4.2.5. The larvae were bled into chilled sterile eppendorf tubes at designated time-points over 60 h post-injection. Serial dilutions of the haemolymph were plated on LB agar supplemented with appropriate antibiotics (described in section 4.2.6) to determine the number of recoverable colony forming units. Haemocytometer counts were also taken to monitor total haemocyte count over the course of the infections.

4.2.8 Growth of *E. coli* carrying *pWEB* or *pWEB-mcf*^{K122} in *M. sexta* and the hosts' total haemocyte count

E. coli carrying either *pWEB* or *pWEB-mcf*^{K122} were grown in 200 ml LB supplemented with 100 μg/ml ampicillin to ~ OD_{600}=0.3 centrifuged and resuspended in 500 μl previously calibrated to contain 1 x 10^{11} cfu/ml equivalent to 1 x 10^{8} cfu/10 μl injected. Injections were carried out as described in section 4.2.5 and the cfu and THC were determined exactly as described in section 4.2.7.

4.2.9 Monitoring *E. coli* clearance and total haemocyte count in *M. sexta* larvae

E. coli carrying *pUC-18* was grown in 200 ml LB supplemented with 100 μg/ml ampicillin to ~ OD_{600} = 0.3 centrifuged and resuspended in 500 μl of GIM previously calibrated to contain 1 x 10^{11} cfu.ml^{-1} equivalent to 1 x 10^{8} cfu/10 μl injected. Serial dilutions were made in GIM and the larvae were injected with 10μl containing 1 x 10^{6} cfu, 1 x 10^{7} cfu or 1 x 10^{8} cfu as described in 4.2.5 and bled at designated times over 24 h post-injection. The cfu and THC were determined over this time as described above in section 4.2.7.

4.3 Results

4.3.1 Time-lapse microscopy of Mcf treated *M. sexta* haemocytes

To examine the effects of Mcf on *M. sexta* larval haemocytes, monolayers of *M. sexta* primary haemocytes monolayers were treated with recombinant Mcf^{W14} expressed in *E. coli*. Differential interference contrast microscopy was used to record images at regular intervals to compile time-lapse movies of the haemocytes with treatment. The movies document the behaviour of *M. sexta* haemocytes as a monolayer on a glass coverslip inverted onto a shallow well of GIM with treatment at 30°C.

Movie 4.1a (Fig 4.1) presents the haemocytes treated with a control cytosolic fraction of *E. coli* carrying *pUC18*. Some prominent differences can be seen between the two main adherent cell types, plasmatocytes and granulocytes, in morphology and mobility. The plasmatocytes are highly motile, actively gliding over the glass coverslip surface adopting numerous short-lived cell shapes. The granulocytes, in contrast, maintain their round morphology with microspikes protruding and retracting transiently. These filopodia extensions appear to 'explore' their surroundings while remaining mostly stationary on the glass coverslip. Healthy haemocyte monolayers maintained these characteristics throughout a12 h incubation when kept under controlled temperature and constant humidity.

Movie 4.1b shows haemocytes treated with cytosolic fractions of *E. coli* expressing Mcf (Fig 4.1). The plasmatocytes lose their motility and spread over the glass surface resulting in a stationary hyper-thin, irregularly shaped cells, eventually exposing minute foci throughout the cell bodies. Some round yet mobile haemocytes are also visible which may be plasmatocytes adopting a round cell morphology, possibly representing the recently described hyperphagocytes. The granulocytes appear unaffected in the first 3-5 h of treatment, but start to undergo membrane blebbing after 4 h treatment. Taken together, the observations from the movies support that Mcf has deleterious effects on haemocytes.

4.3.2 Effects of Mcf on *M. sexta* haemocytes: morphology and actin-cytoskeleton

To investigate if both cell types undergo membrane blebbing with Mcf treatment, individual images from the time-lapse movies were selected (Fig 4.2). Monolayers treated with cytosolic fraction of *E. coli* with either *pUC18* or *pUC18-mcf* are compared, images represent the same view of each monolayer at the start and 6 h into the treatments. The Mcf-treated monolayer at 6 h depicts membrane blebbing in both plasmatocyte and granulocyte, inferring Mcf is active against both of the two major cell types.

The actin cytoskeleton of the treated haemocytes was examined for structural rearrangements, which may allow deduction the mode of action of any actin-targeting toxins. Haemocyte monolayers were treated with cytosolic fraction of *E. coli* carrying either *pUC18* or *pUC18-mcf* for 2 h (before the occurrence of membrane blebbing and cell death), stained with fluorescein-conjugated phalloidin to reveal the haemocytes' actin cytoskeleton and visualised with confocal microscopy (Fig. 4.3), this revealed condensed actin foci throughout the cell body of affected cells. This phenotype appeared comparable to the documented cellular actin rearrangement seen in cells treated with a fungal toxin, cytochalasin D. Cytochalasin D-treated haemocytes display similar actin foci accompanied by the disappearance of actin fibres and lamellopodia irrespective of cell type. Collectively, these data on actin cytoskeleton imply Mcf to cause plasmatocyte-specific actin rearrangements preceding membrane blebbing.

4.3.3 Putative BH3-like domain in Mcf

The observed membrane blebbing of Mcf treated haemocytes is a morphological characteristic of apoptosis, implying Mcf to be pro-apoptotic. Furthermore, the alignment of the amino acid sequences of Mcf from W14 and K122 reveals the presence of a conserved BH3 domain previously described to be pro-apoptotic present in some mammalian proteins. The importance of this hypothetically pro-apoptotic domain was tested using residue specific mutants of Mcf (created by Dr. Gaelle Le Goff, Bath, U.K.). Site-directed mutation of Mcf within the BH3 domain

yielded three single- and one double-residue mutants (Fig 4.4a). Haemocyte monolayers were treated with cytosolic fractions of *E. coli* expressing these recombinant Mcf mutants. The percentage of affected cells undergoing membrane blebbing was recorded for monolayers treated with each mutant (Fig 4.4b). All samples display significantly higher percentage of affected cell as compared to the negative controls of media alone and cytosolic fraction of *E. coli pUC18*. The data show no difference in toxicity amongst the mutants and the wildtype Mcf cytosolic preparations, indicative of the presence of functional pro-apoptotic domain(s) other than the BH3-like domain within the Mcf protein.

4.3.4 Expression of Mcf in *Photorhabdus* W14

Although Daborn *et al.* (2002) demonstrated that Mcf is toxic to *M. sexta* and proposed that it plays a role in the pathogenicity of *P. luminescens* W14, up to now no evidence has been available to show that it is expressed in W14. Several anti-bodies recently created enable this question to be address using Western blot analysis. Supernatants from various growth phases based on OD_{600} of an *in vitro* culture were extracted and analysed for the presence of Mcf using a monoclonal antibody (Fig 4.5). The antibody reacts very strongly against the recombinant Mcf control but did not produce any signal against the *in vitro* samples (Fig 4.5a).

The same anti-MCF-peptide antibody was used in the attempt to detect Mcf in the haemolymph of *M. sexta* larvae injected with either *E. coli* or W14 at 24 and 48 h post-injection (Fig 4.5b). Positive controls of Mcf-expressing *E. coli* and negative controls of *E. coli* p*BAD* vector without insert were used. Despite the high sensitivity of the anti-serum against the denatured full length Mcf visible in the positive control, Mcf was not detected in the haemolymph samples.

4.3.5 Role of Mcf in *Photorhabdus* K122 virulence

Initial screening of a *Photorhabdus* K122 cosmid library by injection and subsequent sequence mining confirmed the presence of a single *mcf* homologue as suggested by previous Southern analysis. A K122 *mcf* knockout ($K122^{MCF-}$ created by Dr. N.R, Waterfield, Bath, U.K.) provides a suitable model to test the role of Mcf in

Photorhabdus virulence by injection. Newly moulted *M. sexta* larvae were injected with either wildtype K122 ($K122^{WT}$) or *mcf*-knockout ($K122^{MCF-}$) at two designated doses of 100 and 1 x 10^5 colony forming units per larvae (cfu/larvae). Mortality rate of the larvae monitored over 7 days were used to calculate the LT_{50} for direct comparison (Fig 4.6). The LT_{50} corresponds to the time at which mortality occurs for 50% of the tested subjects at a given dose. At the lower dose of 100 cfu/larvae, $K122^{MCF-}$ has a LT_{50} of 60.2h, which is significantly longer than $K122^{WT}$ at 40.5h. However, this increase is not evident at the higher dose of $1x10^5$ cfu/larvae where LT_{50} is 22.3 h and 23.2 h for $K122^{WT}$ and $K122^{MCF-}$, respectively.

The *in vitro* growth rates of $K122^{WT}$ and $K122^{MCF-}$ were compared to determine if differential growth rates influenced the increase in LT_{50}. The growth rates of $K122^{WT}$ and $K122^{MCF-}$ in culture were monitored based on OD_{600} at 30°C over 24 h (Fig 4.7a) and culture serial dilutions were plated to determine the cfu at various OD_{600} (Fig 4.7b). Direct comparisons revealed that $K122^{WT}$ and $K122^{MCF-}$ have similar *in vitro* growth rates, with no difference in recovery.

4.3.6 Growth of $K122^{WT}$ and $K122^{MCF-}$ in infected *M. sexta* larvae

Two alternative hypotheses might explain the observed increase in LT_{50} at low dose MCF-knockout ($K122^{MCF-}$) relative to wildtype ($K122^{WT}$). First, there could be an increased susceptibility of $K122^{MCF-}$ to the insect immune responses, rendering the mutant *Photorhabdus* cells less able to combat or circumvent the host immune responses, hindering growth and hence delaying host death. Alternatively, the absence of Mcf could result in reduced virulence of $K122^{MCF-}$ reducing its ability to cause death by direct toxic action. The following experiment was designed to investigate the *in vivo* growth rate of the wildtype and mutant isolates to discriminate between the two possibilities. Newly moulted fifth-instar larvae were injected with 100 cfu of either $K122^{WT}$ or $K122^{MCF-}$ and bled at regular intervals, serial dilutions being plated to recover viable bacteria and thus estimate the *in vivo* growth rate (Fig 4.8a). The total haemocyte count (THC) was also counted and recorded using a haemocytometer (Fig 4.8b). The data reveal that growth rate and THC are similar for $K122^{WT}$ and $K122^{MCF-}$ *in vivo*. This suggests that the increased LT_{50} of $K122^{MCF-}$

is not due to slower bacterial growth, or to diminished ability to deplete the host haemocyte count. It suggests that Mcf may contribute directly to host death by toxaemia.

Clearance of Mcf expressing *E. coli* from *M. sexta* haemolymph

Recombinant Mcf^{K122} expressed in *E. coli* was used to test if Mcf^{K122} alone is sufficient to kill *M. sexta* larvae by injection as was documented for its homologue from W14. $1x10^7$ cells of *E. coli* carrying either a cosmid containing mcf^{K122} or the vector (pWEB) without insert were injected into newly moulted fifth-instar larvae. The larvae were bled at designated time points post-injection and plated to estimate the numbers of colony forming units in the larval haemolymph (Fig 4.9a). The total haemocyte count of each sample was also recorded by haemocytometer counts (Fig 4.9b).

Injection of $1x10^7$ cells of *E. coli* carrying pWEB-mcf^{K122} was lethal for the larvae between 36 – 48 h post-injection while that of pWEB vector was not. However, the rates of bacterial clearance from the haemolymph were similar for *E. coli* pWEB and pWEB-mcf^{K122} indicated by similar decreases in cfu over time. However, the average total haemocyte count profile of the pWEB control injected larvae depicts the typical rise and fall profile as described in figure 2.1b, which was followed by recovery from 18h post-injection to the usual 'resting' haemocyte count at ~2 x 10^6/ insect as previously estimated (Fig. 2.1). This haemocyte recovery was not seen in the larvae injected with pWEB-mcf^{K122} where the THC fell gradually until the death of insects. In summary, recombinant mcf^{K122} expressed in *E. coli* is sufficient to cause death in *M. sexta* at the dose $1x10^7$ cfu / larvae, although either control nor Mcf-expression bacteria were completely cleared from the insect haemolymph by 48h post-injection, the recombinant Mcf^{K122} inhibited haemocyte recovery and caused lethality against the insect host.

4.3.8 Dose-dependant anti-bacteria cellular response in *M. sexta* haemolymph

The clearance of *E. coli* carrying pWEB or pWEB-mcf^{K122} from larval haemolymph was not complete at 48 h post-injection in either case, suggesting insects are able to

tolerate the presence of $1x10^5$ cfu *E. coli* in the haemolymph without notable detrimental effects (Fig. 4.9). I performed a further experiment that aimed to determine if the rate of bacterial clearance from *M. sexta* haemolymph is dependent on the dose of infection agent *E. coli*. Newly moulted fifth-instar larvae were injected with 10 µl containing 1×10^6, 1×10^7 or 1×10^8 cfu of *E. coli* and then incubated at 25°C. Larvae were bled at designated times over 24 h post-injection. The haemolymph samples were plated to determine the number of cfu present (Fig 4.10a). The total haemocyte count of these samples was also determined by haemocytometer counts (Fig 4.10b).

The profiles of *E. coli* clearance on a logarithmic scale appear similar for all larvae regardless of the initial dose of bacteria injected. Clearance was initially rapid and reduced haemolymph bacterial cfu by two orders of magnitude (first 2 h) and then the cfu fell further by about one order of magnitude over the next 22 h (2-24 h). Interestingly, the actual number of bacterial cfu removed from larval haemolymph was 9.9×10^7 in larvae injected with the highest dose (1×10^8 cfu reduced to 1×10^6 cfu in 2 h). This number of viable bacteria removed (9.9×10^7) exceeds both the lower doses injected into other larvae at 1×10^7 and 1×10^6. Intriguingly, the larvae injected with the lower doses failed to clear all the bacteria within 2 h post-injection, but preceded to reduce haemolymph cfu by two orders of magnitude within the first 2 h post-injection. Additionally, the residual bacterial cfu counts in the haemolymph differ by about an order of magnitude corresponding to the injected doses. This suggests the insects' ability to remove bacteria from its haemolymph depends on the dose of bacteria injected.

The profiles of haemocyte numbers are varied between the different doses initially injected. In all cases, there is an initial increase in the number of haemocytes, revealing a lower maximum for a low injected dose and very similar maximum counts for medium and high dose, although the maximum is reached quicker in the case of the medium dose. The haemocyte counts in larvae injected with either low or medium doses returned to the normal total haemocyte count (THC) of $\sim 2.1 \times 10^6$ by 4 and 8 h, respectively and were maintained up to 24 h post-injection. Insects injected with the highest dose of bacteria showed a second increase in the THC at

about 12 h post-injection. This suggests that there may be a threshold number of persisting bacteria in the haemolymph that cases further haemocyte proliferation.

Figure 4.1 Time-lapse movies of *M. sexta* haemocytes under Mcf ^{W14} treatment (Daborn *et al.*, 2002). Monolayers of primary haemocytes on glass coverslips treated with cytosolic fraction of either *pUC18* (movie 4.1a) or *pUC18*-mcf (movie 4.1b) were viewed under differential interference contrast microscopy to record images at regular intervals over time to compile time-lapse movies.

Movie 4.1a show a haemocyte monolayer treated with cytosolic fraction of *E. coli* carrying *pUC18*. Note the motility of plasmatocytes over the glass coverslip resulting in variable cell morphology. In contrast, the granulocytes are generally immobile, however, filopodia can be seen protruding transiently from the cell body although the round morphology is generally maintained.

Movie 4.1b show a haemocyte monolayer treated with cytosolic fraction of *E. coli* carrying *pUC18-mcf*. Note that plasmatocytes lack mobility observed in movie 4.1a, instead they spread flatly over the glass surface. The granulocytes appear as in *pUC18* treatment until 4h into treatment when both cell types start to disintegrate, undergoing membrane blebbing.

Movies can be viewed at http://www.bath.ac.uk/bio-sci/quicktime.html

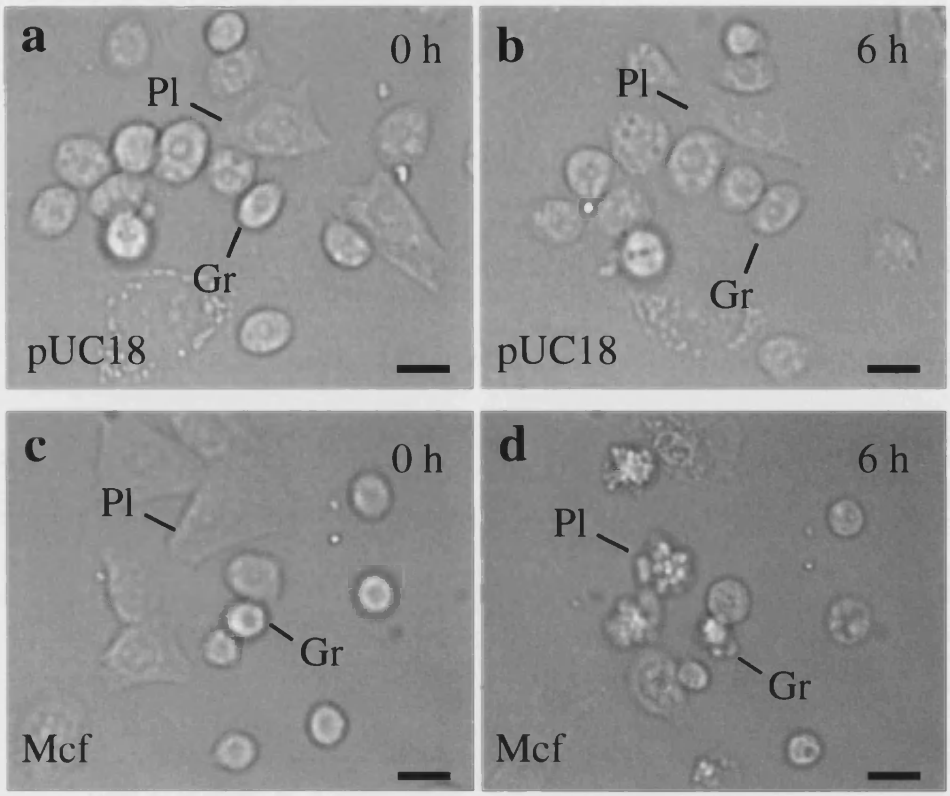


Figure 4.2 *M. sexta* haemocytes treated with Mcf ^{W14} display membrane blebbing in monolayer assay (Daborn *et al.*, 2002). (a and c) Haemocyte monolayers treated with cytosolic fractions of either *pUC18* (a) or *pUC18-mcf* (c) carrying *E. coli* at time beginning of incubation. The same treatments after 6 h (b and d). The same plasmatocyte (Pl) and granulocyte (Gr) are highlighted in the top panels (a and b), Pl displays some changes at 6h while Gr maintained similar morphology throughout treatment. Note the Mcf treated Pl and Gr in (d) have begun to disintegrate by producing a series of rounded blebs, the same corresponding cells at time 0h are highlighted in (c). (Scale bar =10μm)

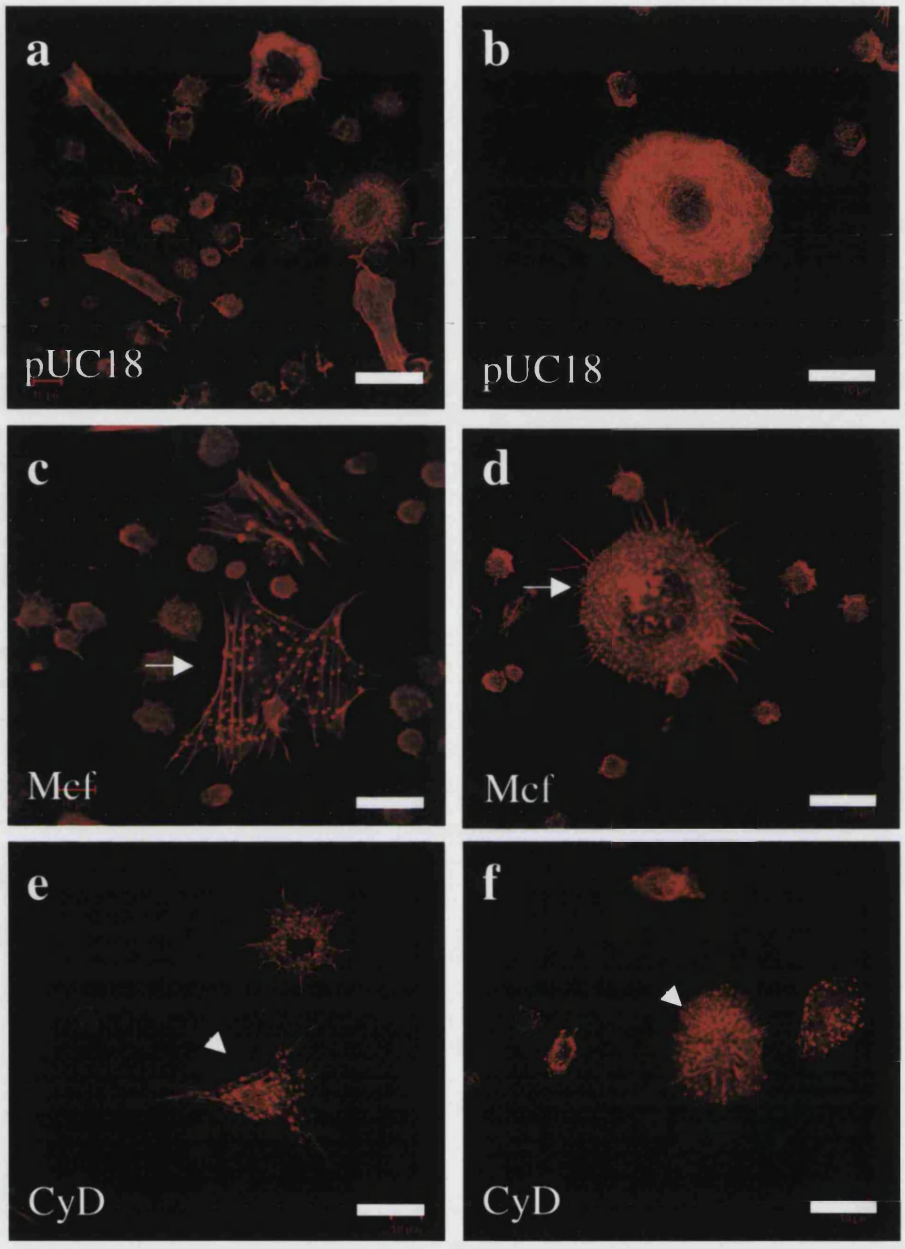
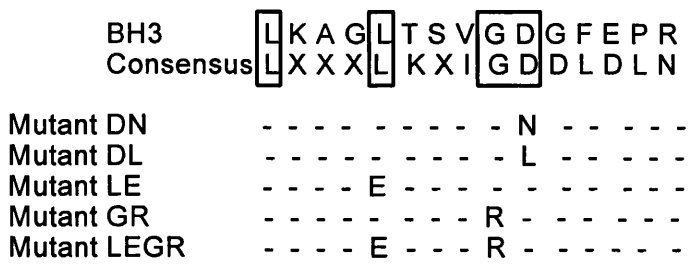


Figure 4.3 Actin cytoskeleton rearrangements in *M. sexta* haemocytes treated with Mcf ^{W14} and cytochalasin D. Confocal images of haemocytes treated with cytocolic fraction from *E. coli pUC18* (a and b), cytosolic fraction from *E. coli pUC18-mcf* (c and d) and cytochalasin D (e and f). Note the presence of punctate actin loci plasmatocytes treated with Mcf (indicated by arrows in c and d) that are absent in the *E. coli pUC18* treated monolayers. Similar condensed actin foci observed in cytochalasin D treated haemocytes (indicated by arrowheads in panels e and f). (Scale bar =20μm)

a



b

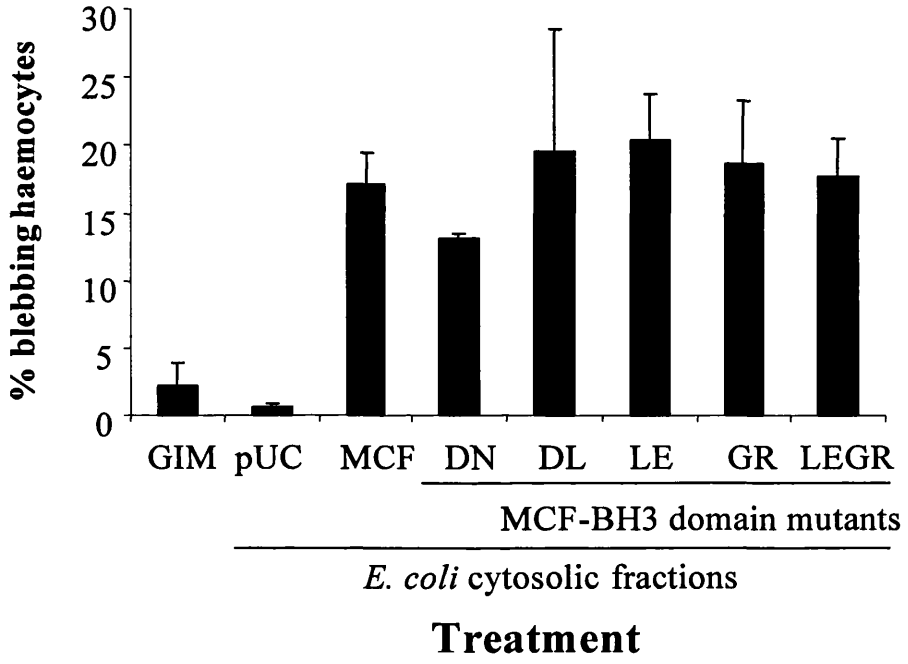
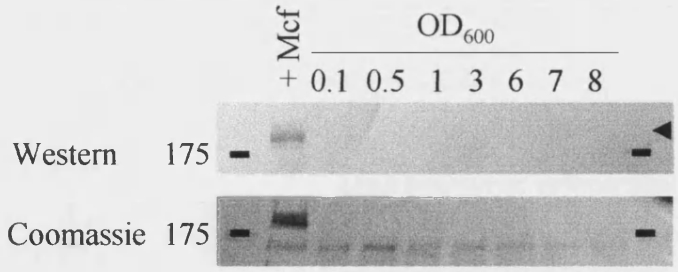


Figure 4.4 Membrane blebbing of haemocytes treated with *E. coli* cytosolic fractions containing Mcf^{W14} with mutated BH3 domains. Fig.4a illustrates the residue replacements by site-directed mutagenesis of Mcf within the hypothetically pro-apoptotic BH3 domain. Haemocytes monolayers treated with the cytosolic preparations were scored for the previously observed membrane blebbing phenotype. Note the percentage of blebbing haemocytes treated with BH domain mutants remain unchanged relative to wildtype Mcf. Data shown represent the mean ± S.D. of >500 cells counted per monolayer per insect, three insects used per treatment.

a – *in vitro culture supernatant*



b – *in vivo larval haemolymph*

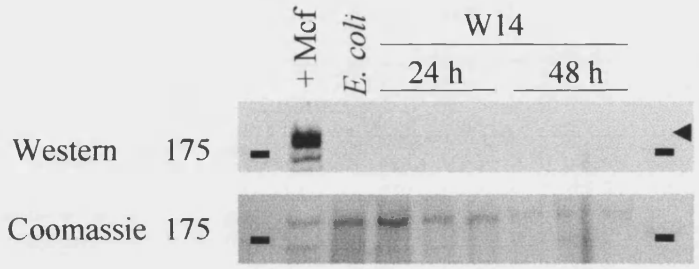


Figure 4.5 Western blot analysis of Mcf^{W14} in *Photorhabdus* W14 *in vitro* and *in vivo*. (a) W14 supernatants from various growth phases of an *in vitro* culture and the protein profiles were resolved by SDS-PAGE and examined using Western blot analysis with a monoclonal anti-Mcf antibody. Arrowheads mark the expected size of full length Mcf above its corresponding Coomassie stained gel. (b) Haemolymph from either *E. coli* or W14 infected *M. sexta* at 24 and 48 h post-injection analysed using a monoclonal anti-Mcf antibody above its corresponding Coomassie stained gel. Note the absence of signal except the positive controls.

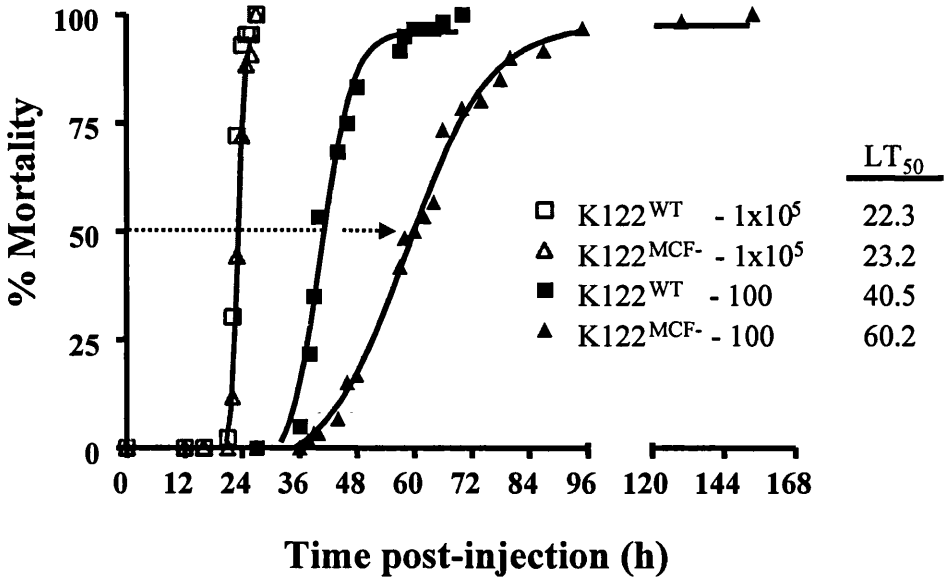


Figure 4.6 LT_{50} determination for *P. temperata* K122 wildtype and K122-MCF knockout mutant. Two doses of exponentially growing K122 were injected into newly moulted fifth instar *M. sexta* larvae. Death of the infected larvae were scored over 7 days to determine the LT_{50}, defined as the time at which 50% of the larvae die at a given dose. K122 wildtype ($K122^{WT}$) and MCF-knockout ($K122^{MCF-}$) have the similar LT_{50} values at the higher dose of 100,000 cfu/larvae. However, note the lower dose of 100 cfu/larvae revealed an increased LT_{50} for the $K122^{MCF-}$ relative to the $K122^{WT}$. Each LT_{50} curve displayed represent the percentage of a minimum of 45 larvae.

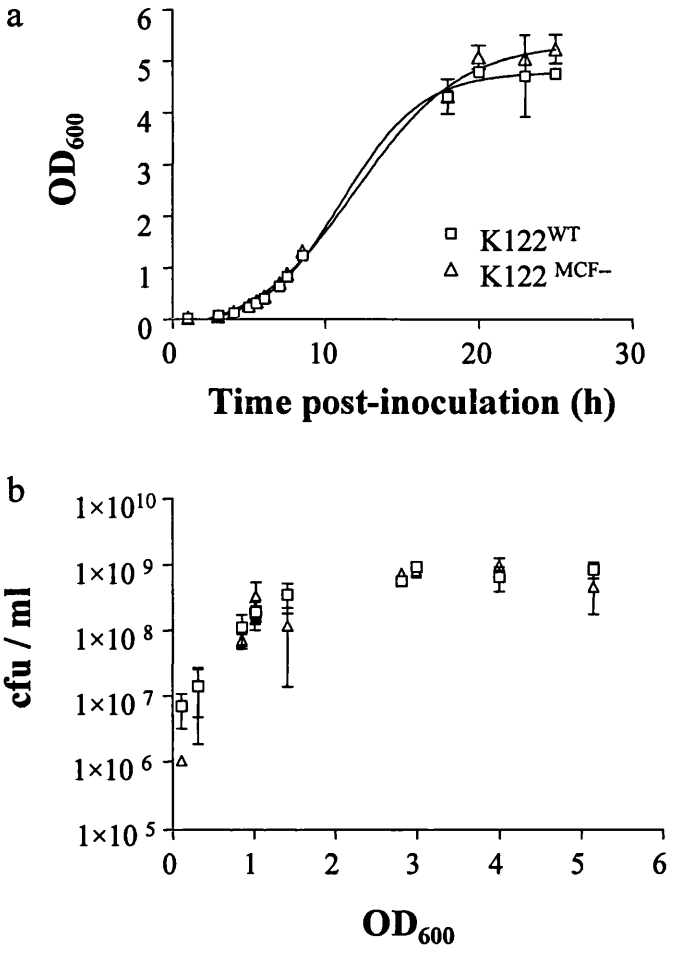


Figure 4.7 *In vitro* growth of *P. temperata* K122 wildtype and K122-MCF knockout mutant. (a) *In vitro* growth of K122 wildtype ($K122^{WT}$) and MCF-knockout ($K122^{MCF-}$) monitored at optical density at 600nm (OD_{600}). (b) Serial dilutions of samples extracted at various OD_{600} and plated to determine the recoverable colony forming units (cfu). Note $K122^{WT}$ and $K122^{MCF-}$ appear to grow at the same rate in culture with similar cfu values over the tested OD range. Data shown in graph 4.7a represents the mean ± S.D. of three independent *in vitro* cultures, data in graph 4.7b represent mean ± S.D. of cfu obtained from a serial dilution range each of three independent cultures.

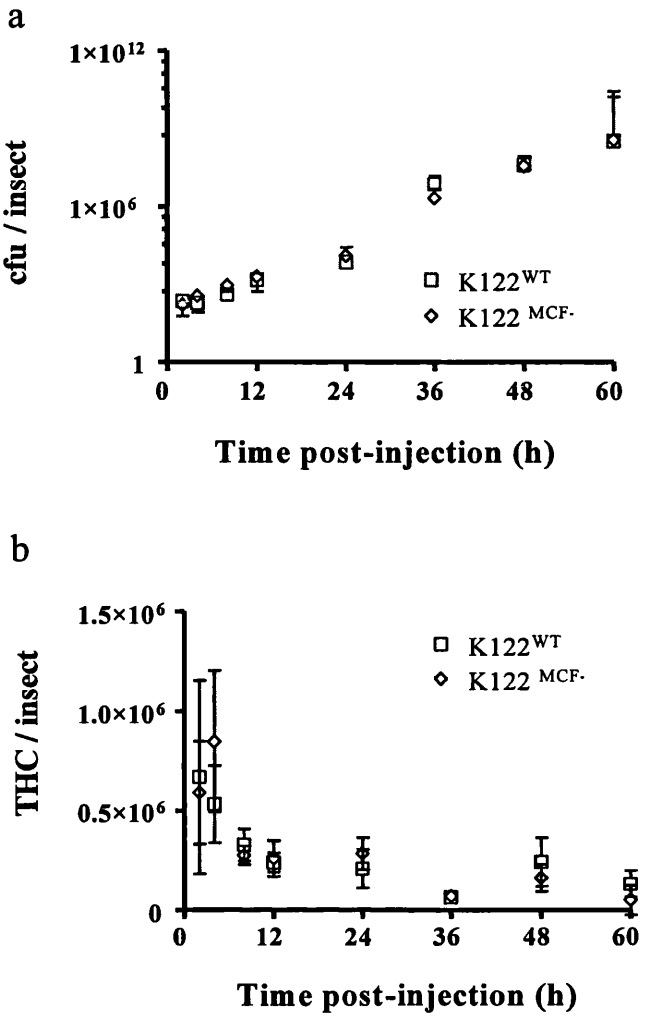


Figure 4.8 *In vivo* growth of *P. temperata* K122 wildtype and K122-MCF knockout mutant in *M. sexta* and the effects on the host total haemocyte count. (a) Colony forming units (cfu) of K122 wildtype ($K122^{WT}$) and MCF-knockout ($K122^{MCF-}$) recoverable from injected larvae over 60h with an initial dose of 100 cfu/larvae. (b) Total haemocyte count (THC) in $K122^{WT}$ and $K122^{MCF-}$ infected larvae monitored at regular time intervals for 60 h post-injection using haemocytometer. Note $K122^{WT}$ and $K122^{MCF-}$ show similar trend in their *in vivo* growth; also steady depletion of haemocyte count. Values for cfu were calculated from plating serial dilutions of haemolymph assuming 320 µl haemolymph/insect (320 ± 86µl, n = 10), data represent mean ± S.E. of six insects/time-point/treatment. THC data shown represent the mean ± S.E. of three samples/insect with six insects/time-point/treatment.

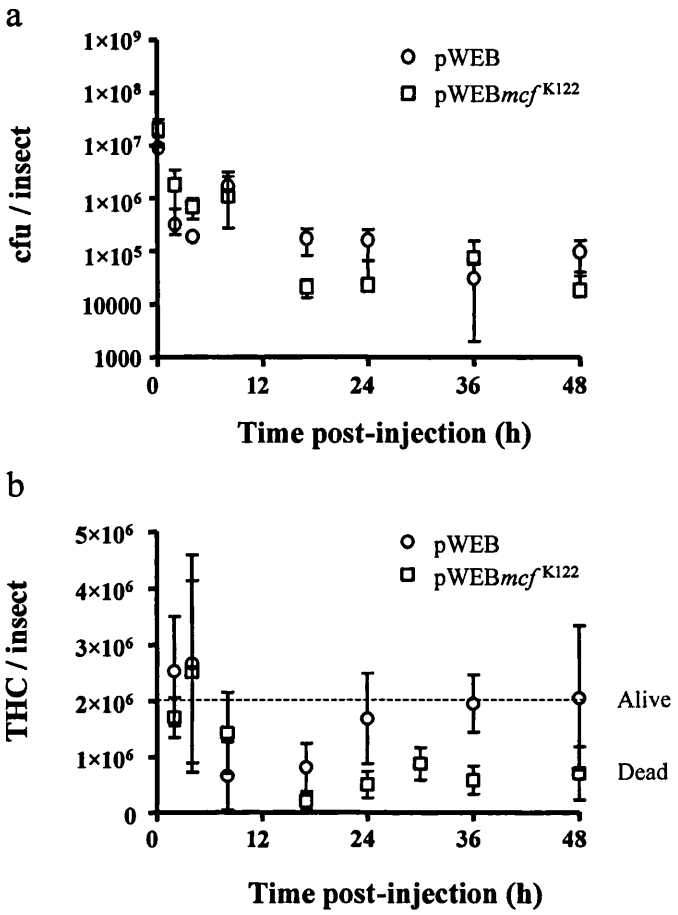


Figure 4.9 *In vivo* cfu of *E. coli* carrying cosmid vector alone or vector with *mcf*^{K122} insert and the effects on the total haemocyte count in *M. sexta* host. (a) Colony forming units (cfu) of *E. coli* carrying either vector (pWEB) or vector with *mcf*^{K122} cosmid containing insert (pWEB-*mcf*^{K122}) in larvae injected with 1 x 10^7 or 3 x 10^7 cfu/larvae, respectively. (b) Total haemocyte count (THC) of larvae injected with ~1 x 10^7 of *E. coli* carrying pWEB or pWEB-*mcf*^{K122} over 48 h post-injection. Note the similar rate of clearance of *E. coli* pWEB and pWEB-*mcf*^{K122} from the larval haemolymph. Also note 3 x 10^7 of *E. coli* pWEB-*mcf*^{K122} is lethal for *M. sexta* larvae, the THC for both treatment reduced at similar rates until 18 h post-injection, at which point THC recovery is observed in *E. coli* pWEB injected larvae but not in that with *mcf*^{K122} cosmid insert. Values for cfu were calculated from plating serial dilutions of haemolymph assuming 320 µl haemolymph/insect (320 ± 86µl, n = 10), data represent mean ± S.E. of four insects/time-point/treatment. THC data shown represent the mean ± S.E. of three samples/insect with four insects/time-point/treatment.

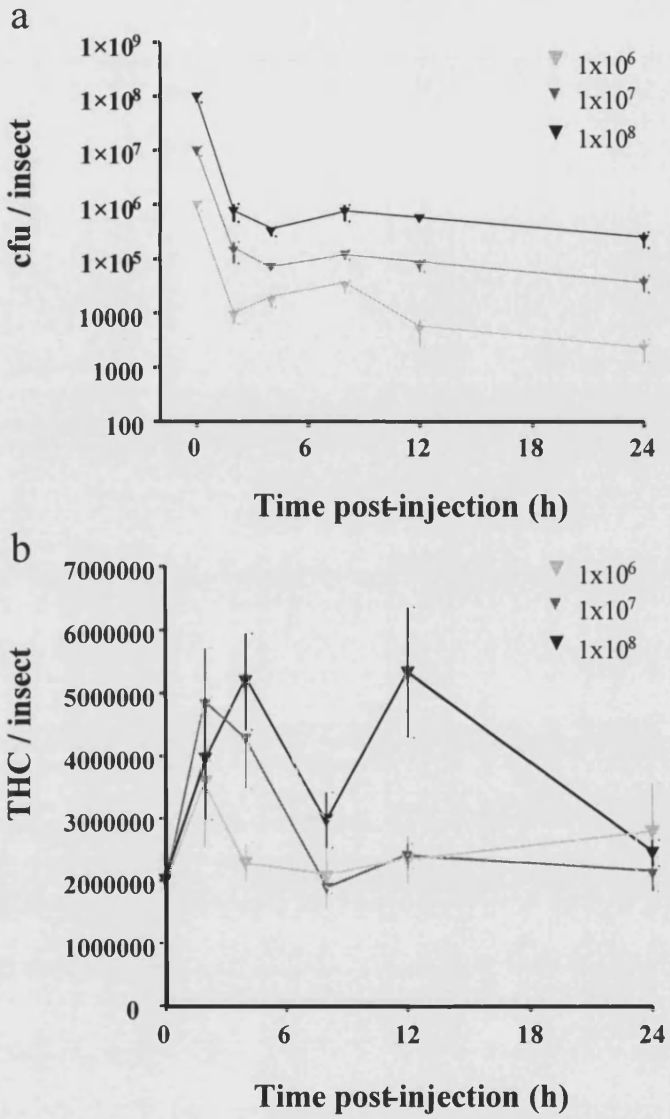


Figure 4.10 Dose-dependent bacterial clearance of *E. coli* from *M. sexta* larval haemocoel. *M. sexta* larvae injected with various doses of *E. coli* were bled to monitor the colony forming units (cfu) (a) and the total haemocyte count (THC) (b) in the larval haemocoel throughout 24 h post-injection. Note majority of bacterial clearance occurs in the first 2 h for all doses studied in a dose-dependent manner. Values for cfu were calculated from plating serial dilutions of haemolymph assuming 320 µl haemolymph/insect (320 ± 86 µl, n = 10), data represent mean ± S.E. of four insects/time-point/treatment. THC data shown represent the mean ± S.E. of three samples/insect with four insects/time-point/treatment.

4.4 Discussion

The expression of Mcf^{W14} in *E. coli* enables the recombinant host to cause a loss of body turgor in *M. sexta* larvae followed by death (Daborn *et al.*, 2002). The treatment of various cultured mammalian cell-lines with Mcf causes the induction of cell death by apoptosis (Dowling *et al.*, 2004). In *P. luminescens* W14, the *mcf* locus is located amongst other haemolysin homologues in a pathogenicity island, *mcf* homologues are also present in all other *Photorhabdus* isolates tested to date, hence *mcf* is speculated to have an important role in combating the host immune system (Daborn *et al.*, 2002). Experiments described in this chapter examine the effects of Mcf^{W14} on *M. sexta* haemocytes through *in vitro* assays and also aim to determine the biological relevance of Mcf in *in vivo* infections against the model host *M. sexta* using recombinant *E. coli* and a *P. temperata* K122 *mcf*-knockout mutant.

Daborn *et al.* (2002) showed that recombinant Mcf^{W14} expressed in *E. coli* is restricted to the cytosol, which is a good resource of the protein. To investigate the effects of Mcf on haemocytes, monolayers were incubated with either Mcf-containing cytosolic fraction or control *E. coli* cytosolic fraction and assessed for characteristics of haemocytes *in vitro* such as viability, mobility, actin phenotype and phagocytic competency (chapter 2). Time-lapse microscopy and actin phenotype examination revealed that Mcf has plasmatocyte-specific effects. Control treatment exposed plasmatocytes are highly mobile adopting varied transient cell shapes gliding across the glass surface while the Mcf treatment inhibited the motility of plasmatocytes which remain stationary and spread out over the glass surface thinly (Fig. 4.1 and Fig. 4.2). Several actin phenotypes have been described in chapters 2 and 3, Mcf could be responsible for the induction of condensed actin foci in plasmatocytes. The fungal toxin cytochalasin D is known to cause a similar phenotype in cultured cell-lines (Fullner and Mekalanos, 2000) and does so by the inhibition of actin polymerisation causing disassembly of fibrous actin (Fig. 4.3). Although the actin phenotypes caused by the two treatments are similar, further investigation would be necessary to confirm this speculative suggested mechanism of action for Mcf.

Control treated granulocytes are round and lack mobility *in vitro* with constant protrusion and retraction of filopodia from the granulocyte cell bodies (Fig. 4.1a). In the first 3 h of Mcf treatment, granulocytes appear unaffected. After 4 to 6 h actin polarity remains normal, but membrane blebbing then occurs (Fig. 4.1b). As *P. luminescens* W14 supernatant treatment disrupts actin polarity in granulocytes, Mcf is unlikely to be responsible for the loss of polarity in granulocytes and is likely to be a plasmatocyte-specific effector. However, figure 4.2 shows that both cell types are affected and undergo membrane blebbing after prolonged exposure of 6 h, which could be explained by a hypothesis of an indirect cause of apoptosis by Mcf since a number of toxin mechanisms lead to apoptosis (Weinrauch and Zychlinsky, 1999). This hypothesis may be possible since *mcf* bears homology to the *A. pleuropneumoniae* Rtx-like toxin export-domain and some Rtx-toxins are able to form non-specific pores by insertion into host cells causing slow leakage and eventual necrosis or apoptosis of host cell (Korostoff *et al.*, 1998). Alternatively, granulocyte death could be an indirect consequence of plasmatocytes death as Pech & Strand (2000) reported that *P. includens* granulocytes undergo apoptosis induced by medium preconditioned by spread plasmatocytes, suggesting that factor(s) released by plasmatocytes can influence granulocyte viability (Pech and Strand, 2000). Therefore further investigation is required to determine if apoptosis is the direct result of toxic Mcf action for both cell types.

To test if the BH3-domain in Mcf is functional, residue-specific mutants of the Mcf BH3-domain were constructed and cytosolic fractions of recombinant *E. coli* were overlaid on haemocyte monolayers to monitor the percentage of apoptotic haemocytes (Fig. 4.4). The data show similar levels of apoptotic haemocytes on monolayers treated with wildtype and BH3-residue mutants, implying the BH3 domain is not responsible for apoptotic cell death. Conversely, similar experiment to assess the effects of Mcf on mammalian cell-lines revealed attenuated toxicity for the LEGR mutant suggesting functional importance for the BH3 domain present (Dowling, unpublished). Expression studies of *mcf*-subclones in mammalian cell-lines are underway and some constructs excluding the BH3-domain also caused apoptotic cell death (Dowling *et al.*, 2004). These contrasting lines of evidence for BH3 functionality require further investigation as it remains possible that Mcf contains more than one functional cytotoxic domain. The expression of the BH3

domain alone may help determine the functional importance of BH3 domain. However, if the structural presentation of the BH3 domain is specific for activity then expression of the BH3 domain alone may not be sufficient to induce cell death. The use of commercially available anti-BH3 peptide may prove to be a useful tool to determine the functional relevance of Mcf in *P. luminescens* W14.

Despite the speculation by Daborn et al. (2002) that Mcf^{W14} has a role in determining virulence, there is still no evidence for the expression of this gene during *P. luminescens* W14 infection *in vivo*. Western blot analyses performed did not detect Mcf in either W14 supernatant *in vitro* or in the haemolymph of W14 bacteria infected *M. sexta* larvae (Fig. 4.5). The lack of detection implies the absence of Mcf in the W14 supernatant; however, the amount of Mcf present may simply be below the detection threshold. More sensitive experiments such as that of quantitative RT-PCR or microarrays would be useful to determine the level of *mcf* transcription as supporting data.

The presence of two close *mcf* homologues in *P. luminescens* W14 renders it unsuitable for knockout studies. Although all *Photorhabdus* isolates tested to date have *mcf1* (Daborn *et al.*, 2002), the isolate *P. temperata* K122 possesses a single *mcf* homologue and therefore, enables the study of *mcf1* single knockout studies. In order to assess the biological relevance of Mcf in *M. sexta* infection, a K122 *mcf*-knockout was constructed (Dr. Waterfield, Bath, U.K.) for direct comparison in virulence (LT_{50}) relative to wildtype (Fig. 4.6). Interestingly, at a higher dose of 1 x 10^5 injected per insect, there is no difference in LT_{50}; the time post-injection at which 50% of the larvae death occurs. However, at a lower dose of 100 cells per insect, the mutant displays an increase in the LT_{50} implying a reduction in virulence. To ensure the differences in LT_{50} is not an artefact of defective *mcf*-mutant growth or reduction in viability over time, *in vitro* (Fig. 4.7a) and *in vivo* (Fig. 4.9a) growth curves and colony forming units at designated growth phase *in vitro* were obtained and confirmed both wildtype and *mcf*- knockout K122 share similar growth rates. These pieces of data indicate that Mcf has a role in *P. temperata* K122 pathogenicity against *M. sexta*.

Injection of Mcf^{K122}-expressing *E. coli* confirms its ability to kill *M. sexta* larvae as had been previously shown with Mcf^{W14} (Daborn *et al.*, 2002). However, similar rates of clearance of Mcf-expressing *E. coli* and the control parent strain of *E. coli* from the larval haemolymph were observed, although larval death was only recorded for Mcf-expression *E. coli* injection. The only observed difference between the two groups of infected larvae was the reduced count of circulating haemocyte in the haemolymph of the insects given Mcf-expressing *E. coli* (Fig. 4.8b). While fluctuations in haemocyte count are recorded immediately upon injection for both treatments, the typical recovery of haemocyte count during bacterial infection which was previously described (chapter 2, Horohov and Dunn, 1982) was not seen for the Mcf-injected larvae. Nevertheless, it is not known if insect death is caused by septicaemia or if the loss of haemocyte count directly affects the viability of the insect.

Given the similar *in vivo* growth rate of wildtype and *mcf*-knockout K122 within the *M. sexta* haemolymph (Fig. 4.9a) and significantly different LT_{50} (Fig. 4.6), larval death is unlikely to be a result of septicaemia. Further, the haemocyte count profile for the wildtype and mutant K122 are also very similar suggesting the absence of Mcf in *in vivo* infection does not affect the total haemocyte count. Additionally, the similar total haemocyte count profile suggests that the sudden lowering of the haemocyte count appears not to affect larval viability. It may be possible that other toxins such as Tcs causing disruption of larval midgut contribute mainly to cause insect host death. However, it should be noted that *P. luminescens* W14 and *P. temperata* K122 are phylogenetically distantly related and have been shown to cause distinct *M. sexta* haemocyte phenotypes (as reported in Chapter 2). Hence, identification and investigation of Mcf homologues in other bacteria may shed light on common role(s), if any, of Mcf in bacterial pathogens.

It is fascinating to note that a *M. sexta* larvae is able to tolerate an *E. coli* bacterial count as high as 1×10^5 colony forming unit bacteria in its haemolymph and survive without apparent detriment (Fig. 4.8). This accentuates the potency of *Photorhabdus*, which is able to cause host death with an initial infection dose of 100 cells (Fig. 4.6). It is also interesting that difference on the LT_{50} between *mcf*-knockout and wildtype K122 is dependent on the number of cells injected (Fig. 4.6).

In vivo experiments were hence designed to investigate the relationship between total haemocyte count fluctuations and number of bacterial injected (Fig. 4.10a). Data reveal the fluctuations in total haemocyte count vary with the number of bacteria injected (Fig. 4.10a). It is interesting to consider when the insect is capable of clearing up to 9.9 x 10^7 bacteria within 2 h, why the insect does not clear all the bacteria within 2 h when injected with 1 x 10^6 cfu of *E. coli*? The haemopoietic lineage of most insects is unclear, the haematopoietic organs in *M. sexta* during embryonic stages and in adult moth are recently reported (Nardi, 2004; Nardi *et al.*, 2003). Granulocytes and plasmatocytes in this insect are suggested to derive from separate lineages which is in conflict with Lavine and Strand (2002) who proposed that plasmatocytes are released into circulation and subsequently differentiate into other cell types including granulocytes (Lavine and Strand, 2002). Unfortunately, the present method used to determine total haemocyte count using haemocytometer does not allow differential cell counts as that relies on the adherence of haemocytes, the property that is affected by the treatment and hence not possible. The use of techniques involving cell type specific monoclonal antibodies or labelled-substrate targeting cell type specific lectins combined with fluorescence-activated cell sorting (FACS) may provide the answer.

Another interesting observation is the second peak in total haemocyte counts in larvae injected with 1 x 10^8 bacteria (Fig. 4.10b). This suggests that there may be a threshold number of bacteria in the haemolymph above which haemocyte are release from sessile state or proliferation of circulating haemocytes promoted. Given the short time period and such large increase in haemocyte count, release of dormant haemocytes or mitotic division of circulating haemocytes is more likely than proliferation of haemocytes from undifferentiated stem cells.

This chapter showed that a single large toxin, Mcf, from both *P. luminescens* W14 and *P. temperata* K122 is capable of inducing *M. sexta* haemocyte cell death *in vitro* and when expressed in non-pathogenic *E. coli* confer lethality by injection. The active toxin domain(s) or the exact mode of action remain obscure and is currently under investigation by using well-established cultured mammalian cell-lines and advanced cellular biological techniques. In addition, data obtained revealed *P. temperata* K122-induced larval mortality is unlikely to be caused by septicaemia and

the transient yet dramatic fluctuations in total haemocyte count does not directly influence larval mortality. Finally, *M. sexta* cellular proliferation to *E. coli* infection is suggested to be concentration dependent, which implies contact-dependent response rather than a global signal such as that of cytokine release in vertebrate immune system. Therefore the present study further knowledge on both bacterial pathogenicity and insect haemocyte response.

Chapter 5

Pdl1, a haemolytic lipase from *Photorhabdus* W14

5.1 Introduction

As mentioned in previously chapters, bacterial pathogens must combat the insect immune response, in order to successfully establish infection. In the case of *Photorhabdus* infections, death of the insect host is absolutely necessary to ensue the continuation of both the bacteria and nematode life cycles. Particular attention is paid to bacterial strategies against phagocytic haemocytes in the context of the thesis. Haemolysins are cytolytic toxins active against blood cells, which are commonly produced by pathogenic bacteria and fungi. Haemolytic microorganisms can be cultured on nutrient agar blood plates to visualise haemolytic phenotype to aid clinical identification.

Unlike *Xenorhabdus*, *Photorhabdus* can lyse mammalian red blood cells when grown on nutrient agar plates (Fischer-Le Saux *et al.*, 1999). *Photorhabdus* displays an unusual phenotype of annular haemolysis with a distinct line of clearance forming a halo 6-12mm from the colony (Akhurst *et al.*, 1996; Farmer *et al.*, 1989). When grown on nutrient agar supplemented with sheep red blood cells this annular haemolysis phenotype, as well as bioluminescence, is used to identify human clinical isolates of *Photorhabdus* (Peel *et al.*, 1999). Annular or discontinuous haemolysis described is rare relative to other haemolysis types; 'α-haemolysis' is partial lysis of blood cells with greening of the medium around the colonies, while completely clear clearance zones is 'β-haemolysis'. Haemolysins are often associated to virulence with varying target specificity and classified by their mode of action, examples of well-characterised haemolysins include the pore-forming *E. coli* HlyA (α-haemolysis; (Welch, 2001), *Streptococcus pyogenes* Streptolysin O (β-haemolysis; (Bhakdi *et al.*, 1996).

Bacillus cereus haemolysin BL (HBL) produces discontinuous haemolysis (Beecher and Wong, 2000). HBL alone is a cooperative toxin, composed of three separately

secreted proteins, which displays activity against a wide range of cell types (Beecher and Wong, 2000). *P. luminescens* subsp. akhurstii strain W14 also displays this unusual annular haemolysis against sheep red blood cells. In order to isolate haemolysins and to investigate their possible role as virulence factors, ~8,000 plasmid clones from a *P. luminescens* W14 genomic library were screened and 12 haemolytic clones were isolated (previous unpublished data). Initial sequence analysis revealed a clone with homology to *mdlB* from *Aspergillus oryzae*, a mono- and di-acylglycerol lipase. The gene was therefore cloned and expressed in *E. coli* for further studies.

Many bacteria produce lipases that hydrolyse esters of glycerol with long-chain fatty acids (Arpigny and Jaeger, 1999; Jaeger *et al.*, 1994). Although the predicted amino acid sequences of lipases are often divergent, these proteins have a conserved serine-protease-like catalytic triad (serine, histidine and aspartate or glutamate) and a consensus pentapeptide, Gly-His-Ser-X-Gly, for prokaryotic lipases (Ogierman *et al.*, 1997). As well as generating considerable biotechnological interest (Jaeger *et al.*, 1999; Jaeger and Reetz, 1998), lipases have the ability to hydrolyse glycolipids (a major component of cell surfaces) hence have been directly implicated in bacterial virulence (Jaeger *et al.*, 1994).

Previous studies of lipase activity in different *Photorhabdus* and *Xenorhabdus* strains (Dunphy *et al.*, 1997; Wang and Dowds, 1993) have distinguished between lipases with a broad substrate activity, which are found in most *Photorhabdus* but only some *Xenorhabdus* strains, and more specific phospatidylcholine-hydrolyzing phospholipases (lecithinases), which are confined to certain *Xenorhabdus* species (Thaler *et al.*, 1998). This distinction in substrate specificity is supported by the observation that mutants lacking the flagellar master operon genes *flhDC*, can make lecithinase but lack lipase and haemolysin activities (Givaudan and Lanois, 2000), suggesting that these phenotypes are encoded by different genes. Further, purified lecinthases from *Xenorhabdus* show a lack of cytolytic activity against sheep red blood cells, or insect haemocytes, supporting a role in lipid metabolism by the bacterial-nematode complex but not in haemolysis (Thaler *et al.*, 1998).

More recent work using haemocytes from *S. littoralis* has shown two distinct haemolytic activities in supernatants of *X. nematophila* (Brillard *et al.*, 2001). The first cytolytic activity appears when bacterial growth enters stationary phase and is active both on insect granulocytes and sheep red blood cells. The second peak of activity occurs later in stationary phase, causing haemolysis of insect plasmatocytes and rabbit red blood cells. Insertional activation of the *fhlD* gene led to loss of the first activity whilst preserving the second, suggesting that they are under independent genetic control (Givaudan and Lanois, 2000).

Therefore, by using *E. coli* expressed Pdl1, this study can confirm the presence of haemolytic factors in W14 and investigate the potential role of the *pdl*1-encoded lipase in the characteristic annular haemolysis of W14. Furthermore, *in vitro* monolayer assays were performed to detect any cytotoxic effects Pdl1 may have against *Manduca sexta* primary haemocytes.

5.2 Material and methods

5.2.1 Bacterial strains and genomic library construction

P. luminescens W14 was used for this study. Genomic DNA was prepared from a 5 ml W14 culture in 2% PP3 (Difco) aerated at 30°C for at least 16 h. The culture was centrifuged at 4 k rpm at 4°C for 15 min and the bacteria pellet was suspended in 561 µl of TE buffer (pH 8), 30 µl 10% SDS with 6 µl of Proteinase K (20 mg/ml) and incubated at 37°C for 90 min 100 µl of 5M NaCl was added and inverted gently to mix followed by 80 µl of preheated CTAB buffer (10% cetyltrimethylammonium bromide in 0.7% NaOH in water) and incubated at 65°C for 10 min. Protein contents were removed using the phenol chloroform extraction. First, 500 µl of 25:24:1 phenol:chloroform:isoamyl-alcahol (IAA) was added and mixed by vortex for 1 min, centrifuged at 13 k rpm at room temperature for 10 min. The aqueous layer was extracted and mixed with 700 µl of 24:1 chloroform:IAA by vortex for 30 sec, centrifuged at 13k rpm, room temperature for 10 min and again the aqueous layer extracted. Finally, the genomic DNA was precipitated with the addition of 420 µl isopropanol, centrifuged at 13k rpm, 4°C for 15 min. The DNA pellet was

washed with 70% ethanol. To remove RNA contaminants, the pellet was suspended in TE with 3 µl of RNaseA (10 mg/ml) at 37°C for 10 min and the above procedure repeated, finally suspending the genomic DNA in 50 µl TE for storage at -20°C until use.

The W14 genomic DNA was partially digested with *Sau*3A (NEB) and fragments between 500 and 2000 bp were extracted from agarose gel and concentrated using a QIAquick Gel Extraction column (QIAGEN). The products were cloned into *Bam*H1 (NEB) digested and calf intestinal alkaline phosphatase (Promega) dephosphorylated pUC-19 vector. The ligation was set up with insert to vector at 3:1 ratio with T4 ligase (Promega) incubated at 16°C overnight. The ligation was electroporated into electro-competent *E. coli XL-1 Blue* (Stratagene) using BioRad Gene Pulser at 2.5 kV with capacitance of 25 µF and 200 Ω resistance. The cells were suspended in LB at 37°C for 1 h to recover and serial dilutions were plated onto LB agar plates supplemented with ampicillin (100 µg/ml) to estimate colony forming units (CFU) per unit volume.

5.2.2 Library screening, cloning and sequencing

Approximately 8000 library clones in *E. coli XL-1 Blue* were screened for haemolytic activity on LB agar plates supplemented with 5% washed sheep red blood cells (OXOID) at 30°C for 48 h. Colonies with halo clearance in the blood agar were screened twice more for consistent haemolytic activity. Nucleotide sequence of positive haemolytic clones was determined on the ABI3700 capillary sequencer using T3 and T7 primers. Sequence data was analysed using DNAstar and subsequently BLAST-X searches were performed against the non-redundant databases of Genbank using NCBI Internet services (previous unpublished data).

5.2.3 *pdl1* cloning and expression

PCR primers were designed based on the initial nucleotide sequences of positive haemolytic library clones. PCR primers 5'-aaggatccatggtgttaatcgttaagaaaa-3' and 5'-cacggatcccaaacatcatgtggtgaaaa-3' were used to amplify the *pdl*1 ORF: 45 cycles

of 94°C for 1 min, 55°C for 1 min, 68°C for 6 min and finally 68°C for 24 min. The 1924bp PCR product was cloned into *Bam*HI restricted pUC-19 then cut with *Hind*III and *Eco*RI and cloned into pBAD-30 vector (kind gift from Mark Blight, France) and electroporated into *E. coli* XL-1 Blue (Constructed by Dr. N. R. Waterfield, U.K.). Positive clones were selected on LB agar supplemented with ampicillin. The plasmid construct was also transformed into *E. coli* K12 strains: MC4100 and CFP201 (MC4100 *sheA*::Tn5-2.1; kind gifts from Dr. I Castillo, Madrid, Spain) and selected with 100 μg/ml ampicillin or 50 μg/ml kanamycin, respectively. Single colonies of pBAD-*pdl*1 were picked to inoculate LB media and 0.2% arabinose was added during exponential phase to induce expression. SDS-PAGE gel analysis was used to confirm expression of protein of correct molecular weight. The haemolytic activity of the clones was confirmed by inoculating single colonies onto LB agar plates supplemented with 5% sheep red blood cells. The clone was also sequenced to ensure it was not mutated.

5.2.4 Liquid haemolysis assay

Filter sterilised cell-free supernatant, washed cells and whole cell lysate of induced and uninduced *E. coli* pBAD-*pdl*1 were obtained from cultures at OD_{600}= 1 to 1.2 (arabinose added at OD_{600}= 0.4 for induced culture). 5% (10 μl) washed sheep red blood cell (sRBC) suspended in PBS was added to 150 μl of treatment sample to make a 200μl total reaction with PBS and incubated at 37°C for 24 h. The reactions were centrifuged at 5 rpm for 5 min to pellet whole red blood cell, the haemoglobin released from lysed sRBC correspond to haemolysis and was quantified by the optical density at 540nm (OD_{540}) of the reaction supernatant. Percentage haemolysis is calculated as [(OD_{540} treatment sample/ OD_{540} total lysis) x100] = Percentage haemolysis (%).

Varying whole haemolysin concentration: volumes between 5-150 μl of whole cell lysate from Pdl expressing *E. coli* were added to PBS to make up a 190 μl mixture, 10 μl of washed sheep red blood cells was also added and experiment carried out as described above.

Varying sheep red blood cells concentration: volumes between 5 – 50 µl of washed sheep red blood cells were added to PBS to make up to total volume of 50 µl. This was mixed with 150 µl of washed cells of Pdl1 expressing *E. coli* and experiment carried out as described above.

5.2.5 Lipase activity assay

P. luminescens W14 and *E. coli* pBAD-*pdl*1 (with or without arabinose induction) were tested for lipase activity by inoculating appropriate nutrient agar supplemented with various lipase substrates. (i) 10% (vol/vol) emulsified Tween-20 or Tween-80 (Sigma) was added to molten LB-agar with 1mM $CaCl_2$ cooled to ~55°C before pouring. (ii) 10% (vol/vol) emulsified tributyrin (Sigma) and 0.1% (wt/vol) gum arabic was added to molten LB-agar before pouring. The plates were incubated at 30°C for 48 h and lipase activity is indicated by halos of insoluble free fatty acids released from the Tween substrates and clear halos should appear around the colonies with trybutyrin as substrate.

Washed cells of *P. luminescens* W14 and *E. coli* pBAD-*pdl*1 (induced and uninduced) were tested in liquid lipase assays against (i) *p*-nitrophenyl caproate (pNPC; Sigma) and (ii) *p*-nitrophenyl palmitate (p-NPP; Sigma). Substrate solutions were prepared as follows: (i) 23.7 mg of pNPC was dissolved in 10 ml of 2-propanol (ii) 30 mg pNPP was dissolved in 10 ml of 2-propanol at 60°C, and added to 90 ml of sodium phosphate buffer (pH 8) supplemented with 207 mg of sodium deoxycholate and 100 mg of gum arabic. For the reactions, 50 µl of washed cells was added to the 950 µl substrate solutions and incubated at 37°C for 30 min. Lipase activity is quantified by optical density at 410 nm (OD_{410}).

5.2.6 Sub-cellular fractionation

Washed cells from arabinose-induced and uninduced pBAD-*pdl*1 culture was suspended in native cell lysis buffer (Sambrook *et al.*, 1989) and chilled on ice for 30 min and sonicated at 12 µm (10s on/10s off). The total lysate was centrifuged at 100,000 g for 2 h and the supernatant contain the cytosolic / periplasmic fraction and

pellet resuspended as the membranous fraction. The protein samples were mixed with 1 volume of 2x loading buffer and incubated at 95° for 5 min, then on ice. The protein profiles were resolved in a 10% SDS-PAGE gel at 120 mV for 90 min (section 2.2.5).

5.2.7 *E. coli* pBAD-*pdl*1 toxicity against *M. sexta* larvae

Recombinant *E. coli* carrying pBAD-30 vector only and pBAD-*pdl*1 were grown to OD_{600} of 0.6 in the presence of 0.2% arabinose, the cells were washed in PBS and resuspended at 1 x 10^7, 1 x 10^9 and 1 x 10^{11}/ml. Fifth instar larvae were chilled, surface sterilized and injected with 10 µl of the washed cells delivering 10^5, 10^7 and 10^9 cells per 10 µl into each larvae, respectively. The larvae were kept at 25°C for 4 days to score for mortality. Three larvae were injected for each concentration per treatment.

5.2.8 *In vitro* haemocyte mortality and actin morphology

Haemocyte monolayers prepared as in section 2.2.3 and haemocyte viability assay as described in section 2.2.4. Trypan blue stained and total cell counts were visualized and recorded with light microscope at 200x magnification. Data shown were calculated from counts of at least 500 cells / monolayer / insect and three insects per treatment.

5.2.9 Phagocytosis assay

Haemocyte monolayers for *in vitro* assays were prepared as described in section 2.2.3 and phagocytosis assays conducted according to section 2.2.8. The haemocytes were stained with TRITC-phalloidin as in section 2.2.6. Labeled haemocytes were visualized using a Zeiss LSM-510 confocal microscope and percentage of phagocytic haemocytes determined by counting TRITC-stained haemocytes co-localised with the *E. coli*-GFP in relation to total haemocyte count. Data shown were calculated from counts of at least 500 cells / monolayer / insect and three insects per treatment.

5.2.10 Site-directed knock-out of *pdl*1 in W14 - suicide plasmid construction

The knockout approach requires integration of a suicide plasmid at the site directed target of the host bacteria chromosomal DNA. The suicide vector must contain the target gene (*pdl*1) interrupted by the insertion of an antibiotic cassette for final selection. It also requires *sacB* gene from *Bacillus subtilis* encoding levansucrase, which is lethal to many gram negative bacteria and hence enables selection for gain of sucrose-sensitivity is indicative of plasmid integration. The cloning strategy for suicide plasmid involves various steps, in all cases unless otherwise stated enzymes were purchased from NEB, ligations were electroporated into electro-competent *E. coli XL-1 Blue* (Stratagene) using BioRad Gene Pulser at 2.5 kV with capacitance of 25 μF and 200 Ω resistance settings. The cells were suspended in LB at 37°C for 1 h to recover and selected on LB plates with the appropriate antibiotics and Mini-prep kit (QIAGEN) was used for all DNA purification steps.

The plasmid pUC19-*pdl*1 (section 5.2.3) was digested with *Eco*RV situated in the middle of *pdl*1 locus, dephosphorylated with CIP (Promega) and ligated to *Sma*1-cut streptomycin cassette. Positive clones were selected with 100 μg/ml ampicillin and 10 μg/ml streptomycin and insert orientation confirmed by restriction analysis with *Hind*III. The product pUC19-*pdl*1::strp was then cut with *Pst*1 and dephosphorylated and ligated to *Pst*1 cut *sacB* from pKS115 (kind gift from Dr. N. R. Waterfield, U.K.). Restriction analysis with *Hind*III or *Pst*1 to confirm cloning of ampicillin and streptomycin resistant clones.

5.2.11 Electroporation of electro-competent W14 and selection

Fresh electro-competent W14 is required for transformation of plasmid. 50 ml of exponential phase culture with optical density at 600 nm (OD_{600}) between 0.4 and 0.6 is chilled on ice fro 30 min. Obtain cell pellet by centrifugation at 10 k rpm at 4°C for 30 min. Then remove supernatant and suspend cells in original volume of chilled sterile dH_2O and repeat centrifugation step. Decant supernatant and suspend

cells in 5 ml of chilled sterile 10% (vol/vol) glycerol and repeat centrifugation step. Suspend the cells once more and repeat centrifugation step. Finally suspend the cells in 1/400 of original volume of chilled sterile 10% glycerol. Aliquot cells and store on ice until use.

Electroporation of pUC19-*pdl*1::strp into W14 using BioRad Gene Pulser at 2.5 kV with capacitance of 25 μF and 200 Ω resistance. The cells were suspended in 2% PP3 at 30°C for 1 h to recover and selected on PP3 plates with the 100 μg/ml ampicillin and 10 μg/ml streptomycin. Viable clones were picked and grown in 2% PP3 without selection at 30°C for 48 h and plated on to PP3 agar with 10 μg/ml streptomycin. Colonies were then patched onto PP3 agar plates with various combinations of supplements to select for ampicillin-sensitive, sucrose-tolerant and streptomycin resistant indicative of true mutants.

5.3 Results

5.3.1 Predicted amino acid sequence of Pdl1 and Pdl2

In order to isolate haemolytic clones from W14, a size-selected genomic library was transformed into *E. coli XL1-Blue* and screened on nutrient agar plates for haemolysis against sheep red blood cell. The initial screen of ~8,000 clones uncovered 12 haemolytic clones, subsequent sequence analysis revealed one clone to have sequence identical to an open reading frame predicting a *Photorhabdus diacylglycerol lipase*-like (*pdl*) gene. This gene, termed *pdl1*, lies downstream of *tcdA1B1* within the recently described *tcd* pathogenicity island of W14. A second *pdl*-like gene, *pdl2*, is also present in this genomic island adjacent to a different block of *tcdAB*-like genes (Waterfield *et al.*, 2002a). Interestingly, these genes are also found in the closely related strain TT01. The two *pdl* genes predict very similar proteins, Pdl1 and Pdl2, with 78% amino acid identity and 85% similarity (Table 5.1) and similar molecular weights at 73.0 and 73.6 kDa, respectively.

The predicted amino acid sequence of *pdl1* and *pdl2* were aligned with two other lipase-like products from *mdlB* gene from *Aspergillus oryzae*, a mono- and di-acylglycerol lipase (designated L2) and a hypothetical open reading frame from *Vibrio cholerae* (Fig. 5.1). The alignment revealed that Pdl1 and Pdl2 possess the three conserved predicted active site residues (Ser, Asp, His) and the consensus pentapeptide Gly-His-Ser-X-Gly common in active lipases. Together, the data suggests the predicted Pdl1 could encode for an active lipase with haemolytic activity, moreover its homology to the hypothetical ORF in *V. cholerae*, another known pathogen, offers the potential of a virulence factor.

5.3.2 Pdl1 lysis of sheep red blood cell on agar plates

To confirm that the predicted Pdl1 accounts for the haemolysis observed in the screens, the full-length *pdl*1 gene was cloned into an inducible expression vector pBAD-30. *E. coli* XL-1 Blue carrying pBAD-*pdl1* grown on nutrient agar blood plates with and without arabinose at 37°C for 48 h showed arabinose-induction

dependent haemolysis (Fig. 5.2b + c). The haemolysis observed is diffused around the colonies is β-haemolysis-like without greening in the surrounding agar. This is in contrast to the annular haemolysis displayed by *P. luminescens* W14 when grown on PP3 blood plates, where the zones of red blood cell lysis are remote from the colony growth (Fig. 5.2a).

A reduced growth rate was noted for the arabinose-induced *E. coli* carrying pBAD-*pdl1* relative to the uninduced (data not shown), suggesting detrimental effects either of the Pdl1 expressed or the recombinant expression system. Bacteria-overlay halo assays yielded data in support of Pdl1 with bacteriocidal properties, however, these experimental results were inconsistent and requires further investigation. Further, the use of *E. coli* XL-1 Blue carrying pBAD alone with arabinose would eliminate the inducible-inducible expression system as being the cause in bacterial growth rate.

To exclude the possibility that the haemolysis observed is caused by an activated silent h(a)emolysin A (*sheA*), a cryptic haemolysin in *E. coli* (del Castillo *et al.*, 1997), the same construct was transformed into *E. coli* K12 MC4100 and CFP201, its *sheA* mutant derivative of MC4100 and tested for haemolysis. Both strains carrying pBAD-*pdl1* grown on nutrient agar blood plates displayed *sheA*-independent haemolysis in the presence of arabinose (data not shown). Collectively these results confirm Pdl1 to be the causative agent of the sheep red blood cell lysis independent of any cryptic haemolysins in *E. coli* host. Interestingly, this lysis was not annular, suggesting other bacterial gene products are also important in this unusual phenotype.

5.3.3 Pdl1 lysis of sheep red blood cell in liquid haemolysis assay

As some haemolysins only exhibit haemolytic activity on blood plates or in liquid assays (Beem *et al.*, 1999), Pdl1 was also tested for haemolytic activity in a liquid haemolysis assay. Cell-free supernatant and washed cells of W14, K122 and induced *E. coli* XL-1 Blue carrying pBAD-*pdl*1 were tested. The percentage haemolysis was determined by the amount of haemoglobin released measured by optical density at 540nm (OD_{540}) relative to total lysis of a control sample. Interestingly, neither cells nor supernatant of either *Photorhabdus* strain displayed

haemolysis in the liquid assay. Further, only washed cells of *E. coli* expressing Pdl1 showed haemolysis significantly higher than control (Fig. 5.3). These results suggest Pdl1 to be a cell-associated haemolysin when expressed in *E. coli*, however W14 was negative for haemolysis in the liquid assay implying that Pdl1 is not expressed or haemolysis is suppressed in W14.

5.3.4 Liquid haemolysis with varying Pdl1 and red blood cell concentration

Pdl1 is likely to be cell associated as washed cells of *E. coli* expressing Pdl1 display haemolysis in the liquid haemolysis assay (Fig. 5.3). This was confirmed when whole cell lysate of Pdl-expressing *E. coli* was tested in the liquid assay (Fig. 5.4a). Using whole cell lysate as Pdl1 source, it may be possible to deduce the mode of action by observing changes in the level of haemolysis with varying amounts of haemolysin (Pdl1) and substrate sheep red blood cells. For example, it may be possible to distinguish pore-forming activity from enzymatic catalysis. Total reactions of 200 µl containing 5, 10, 25, 50 or 100 µl of whole cell lysate caused an average haemolysis between 46 and 53%, and 150 µl caused 83 ± 3% (Fig. 5.4b). With the exception of the 150 µl reaction, similar levels of haemolysis were observed over increasing amount of haemolysin. This suggests the mode of lysis to be enzymatic, whereby the rate of reaction in the presence of excess enzyme is limited by the amount of available substrate.

Conversely, assays with varying amounts of sheep red blood cells of between 5 and 50 µl displayed a trend of decreasing percentage haemolysis with increasing red blood cells available (Fig. 5.4c). This result suggests the mode of lysis to be pore-formation, where addition of more cells would dilute the haemolysin, and limit the opportunity for co-operative interactions between haemolysin molecules.

5.3.5 Subcellular localisation of Pdl1

In order to examine the distribution of Pdl1 within the recombinant *E. coli*, cells of Pdl1 expressing *E. coli* were fractionated to obtain whole cell lysate, cytosol and membrane fractions. SDS-PAGE analysis revealed that Pdl1 is produced in the

recombinant *E. coli* as a 73 kDa protein and is abundant in the cytosol (Fig. 5.5). This raises interesting questions of how Pdl1 is exported in *Photorhabdus* and if contact between the haemolysin and target cell is a prerequisite for lysis.

5.3.6 General lipase activity

Since Pdl1 has homology to *mdlB* gene from *Aspergillus oryzae* and is predicted to have the conserved lipase active site residues, standard lipase tests were carried out. The substrates Tween-20, Tween-80 and tributyrin were added to nutrient agar plates and hydrolysis by active lipases creates a halo around the lipase positive colonies. W14 is positive for lipase activity against Tween-20 and Tween-80 but not tributyrin, while the recombinant *E. coli* showed arabinose-independent Tween-20 hydrolysis but is negative for all other substrates (Table 5.2).

5.3.7 Pdl toxicity against *M. sexta* haemocytes *in vitro*

As Pdl1 is abundant in the cytosol of the recombinant *E. coli*, this fraction was used in an *in vitro* assay to detect any toxicity against primary *M. sexta* haemocytes. Haemocyte monolayers prepared were incubated with the cytosolic fractions of arabinose induced *E. coli* carrying pBAD alone and pBAD-*pld*1. Haemocyte viability was examined using trypan blue, a dye that is excluded by viable cells, hence staining dead cells blue. There was no significant difference in haemocyte viability between the treatments under the experimental conditions used (Table 5.3).

Further, the haemocytes treated with cytosolic fractions of Pdl1-expressing *E. coli* showed no distinct actin cytoskeleton morphology compared to haemocytes treated with *E. coli* control cytosolic extracts (data not shown). Finally, the ability of the haemocytes to phagocytose GFP-labelled *E. coli* in the presence of Pdl1 was also unaffected relative to the *E. coli*-pBAD alone control. Together the data show that Pdl1 is not cytotoxic against haemocytes of *M. sexta*.

Figure 5.1 Alignment of the predicted amino acid sequences of two *Photorhabdus* lipase genes, *pdl1* and *pdl2* (Genbank AY144119), with predicted products of the *A. oryzae mdlB* gene (Genbank D85895) and a *V. cholerae* hypothetical open reading frame, VC1418 (Genbank AE004220). The presence of the presumptive serine protease-like catalytic triad (S, D and H) is highlighted (red) alongside the conserved pentapeptide GHSXG (yellow) common to lipases and lipoprotein lipases.

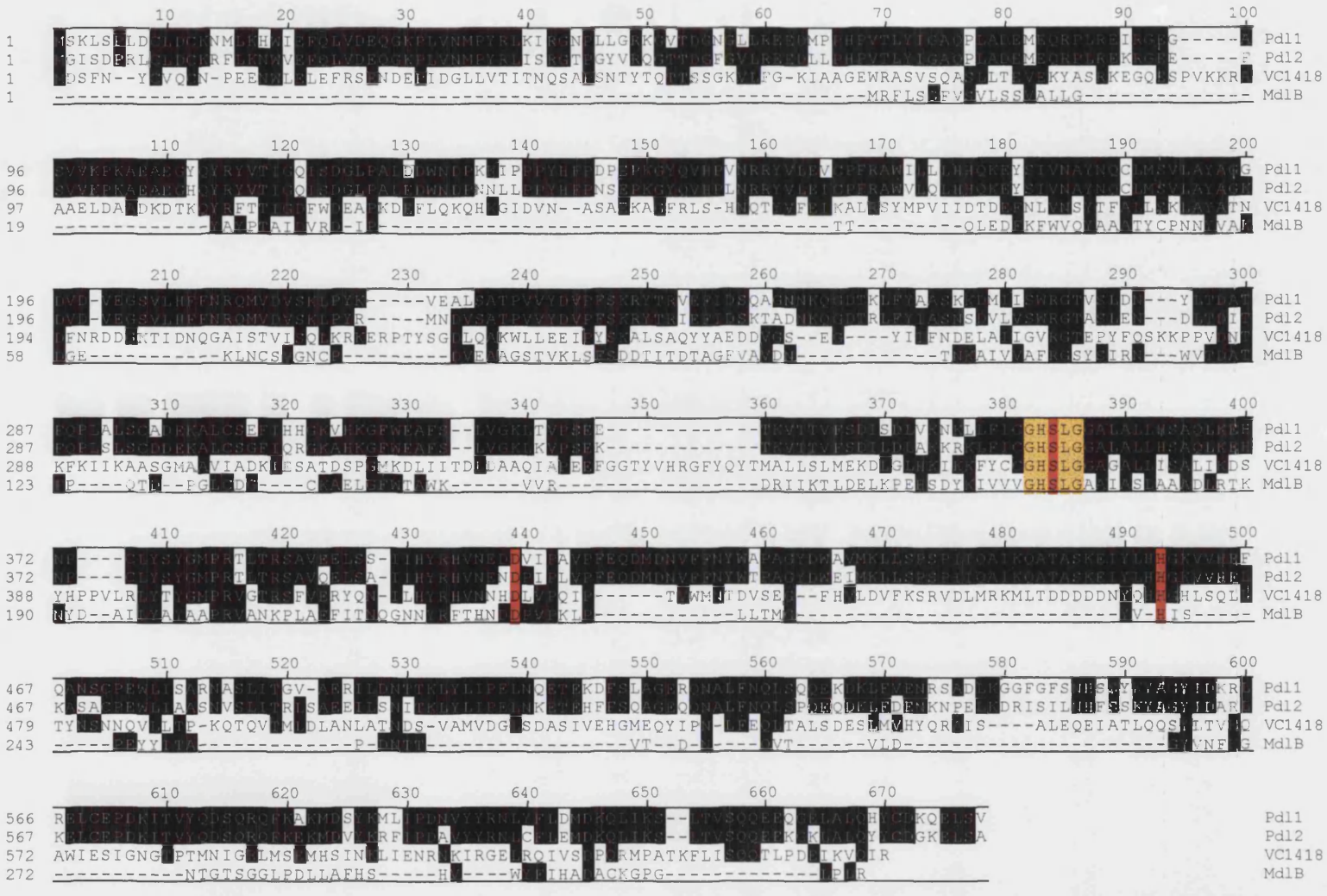

Pdl1
Pdl2
VC1418
MdlB

Table 5.1 Percentage amino acid similarity and identity (in parentheses) between the two predicted *Photorhabdus* diacylglycerol lipases, Pdl1 and Pdl2, MdlB from *A. oryzae* and VC1418 from *V. cholerae*.

		Identity			
		Pdl1	Pdl2	VC1418	MdlB
Similarity	Pdl1	100	(78)	(22)	(24)
	Pdl2	85	100	(23)	(22)
	VC1418	35	36	100	(28)
	MdlB	43	41	48	100

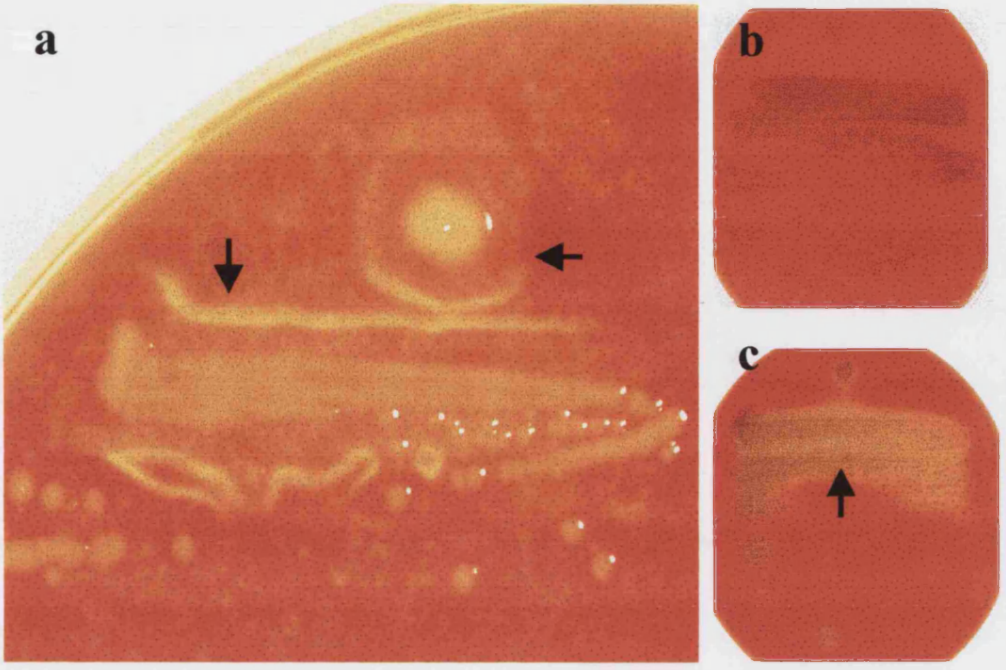


Figure 5.2 Differing haemolytic phenotypes of *P. luminescens* W14 and Pdl1 expressing *E. coli* against sheep red blood cells. Comparison of the haemolytic phenotypes displayed by (a) *P. luminescens* W14 and (b) uninduced and (c) arabinose induced *E. coli* expressing Pdl1 on agar plates containing sheep red blood cells. Note the complex annular haemolysis shown by *P. luminescens* (arrows in panel a) versus the simple zone of haemolysis surrounding the induced *E. coli* expressing Pdl1 (arrow in panel c).

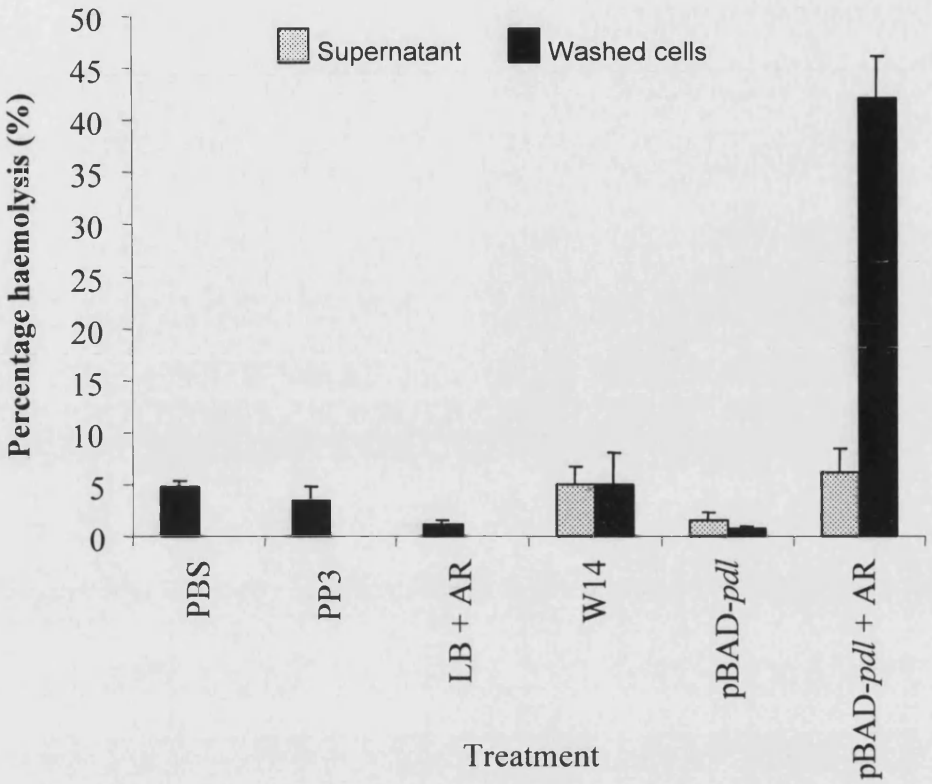


Figure 5.3 Haemolysis of sheep red blood cells by supernatant and cell fractions of *P. luminescens* W14 and Pdl1 expressing *E. coli* in liquid assay. A series of controls (PBS, phosphate buffered saline; PP3, proteose peptone no.3; LB + AR, LB plus arabinose; pBAD-*pdl*1, uninduced pBAD-*pdl*1 expressing *E. coli*) compared to supernatants and washed cells of W14 and Pdl1 expressing *E. coli*. Results are expressed as percent haemolysis, where 100% corresponds to the amount of haemoglobin released by complete lysis of the red blood cells. Note only one treatment sample, washed cells of Pdl1 expressing *E. coli*, showed significant haemolysis in this liquid assay. Data represent mean ± S.D. of triplicate samples.

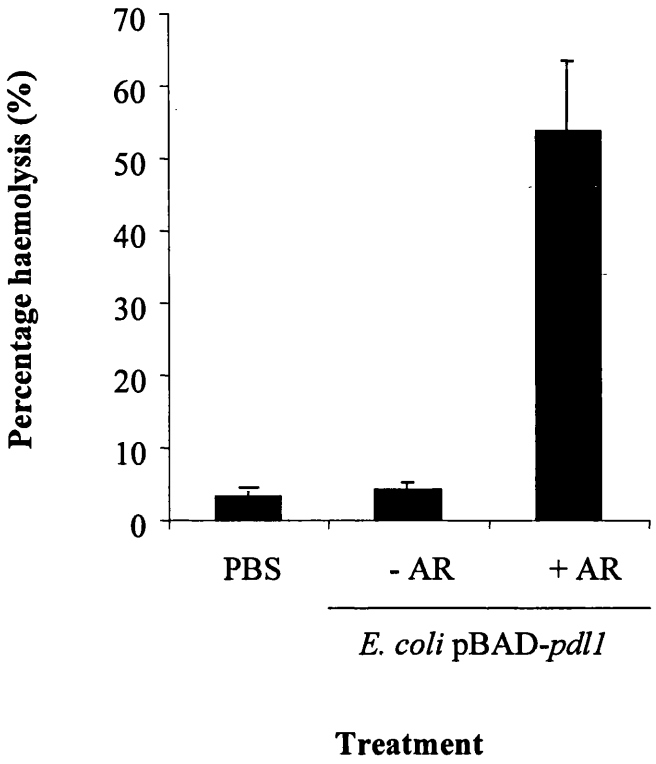


Figure 5.4a Haemolysis of sheep red blood cells by whole cell lysate from Pdl1 expressing *E. coli* in a liquid assay. Controls (PBS, phosphate buffered saline; *E. coli* pBAD-*pdl* -AR, uninduced pBAD-*pdl* expressing *E. coli*) compared to whole cell lysate from induced pBAD-*pdl* expressing *E. coli*. Results are expressed as percent haemolysis, where 100% corresponds to the amount of haemoglobin released by complete lysis of the red blood cells. Note whole cell lysate of Pdl expressing *E. coli* cause significantly higher haemolysis relative to the same fraction from the uninduced control. Data represent mean ± S.D. of nine samples.

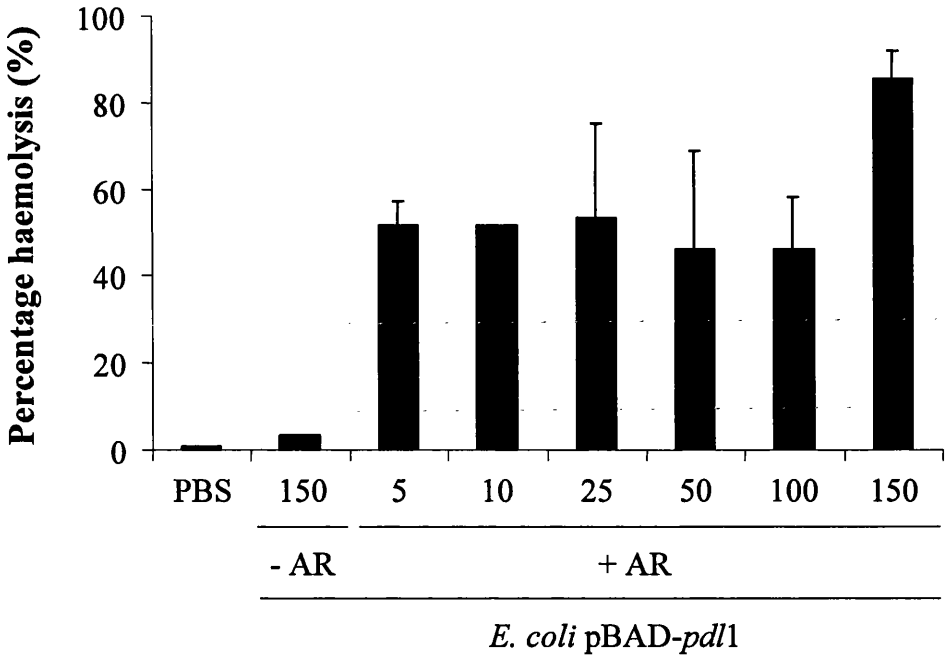


Figure 5.4b Liquid haemolysis of sheep red blood cells with varying concentrations of whole cell lysate from Pdl1 expressing *E. coli*. Various amounts of whole cell lysate from *E. coli* pBAD-*pdl* +AR (induced pBAD-*pdl1* expressing *E. coli*) compared PBS (phosphate buffered saline) and whole cell lysate from *E. coli* pBAD-*pdl* +AR (uninduced *E. coli* pBAD-*pdl1*) controls in haemolysis against sheep red blood cells. Results are expressed as percent haemolysis, where 100% corresponds to the amount of haemoglobin released by complete lysis of the red blood cells. Note increasing concentrations of *E. coli* pBAD-*pdl* +AR lysate resulted in generally unaltered percentage haemolysis, with the exception of *E. coli* pBAD-*pdl* +AR at 150µl. Data represent mean ± S.D. of triplicate samples.

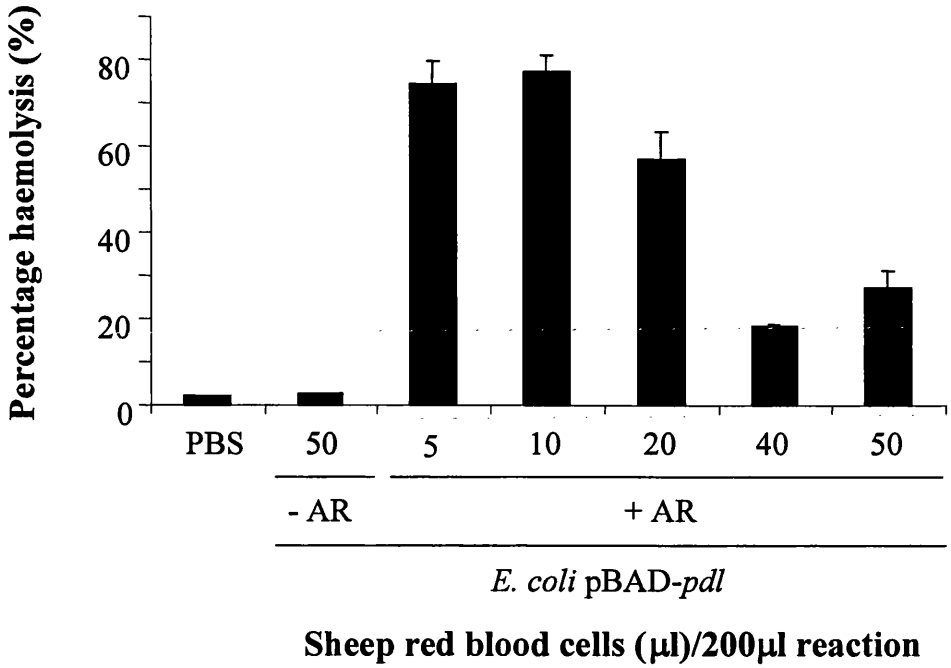


Figure 5.4c Liquid haemolysis by whole cell lysate from Pdl1 expressing *E. coli* against a range of sheep red blood cell concentrations. PBS (phosphate buffered saline) and whole cell lysate from *E. coli* pBAD-*pdl* -AR (uninduced *E. coli* pBAD-*pdl1*) controls and whole cell lysate from *E. coli* pBAD-*pdl* +AR (induced pBAD-*pdl1* expressing *E. coli*) compared in haemolysis against varying concentrations of sheep red blood cells. Results are expressed as percent haemolysis, where 100% corresponds to the amount of haemoglobin released by complete lysis of the red blood cells. Note decreasing percentage haemolysis with increasing concentration of sheep red blood cells. Data represent mean ± S.D. of triplicate samples.

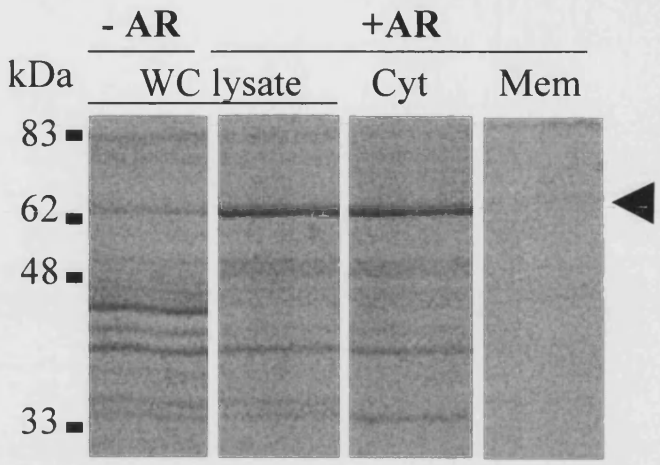


Figure 5.5 SDS-PAGE analysis of recombinant Pdl1 in *E. coli*. Gel showing the presence of the expected 73 kDa protein in whole cell lysate (WC lysate) from arabinose induced (+AR) *E. coli* pBAD-*pdl* and its absence in the same extract from uninduced (-AR) *E. coli* pBAD-*pdl* (lanes 1 and 2). Lanes 3 and 4 show localization of the recombinant Pdl1 to the cytosolic fraction (Cyt) of pBAD-*pdl* containing *E. coli* and its absence from the associated membrane fraction (Mem).

Table 5.2 Active lipase activity of *P. luminescens* W14 and Pdl1 expressing *E. coli* against tributyrin, Tween-20 and Tween-80 on nutrient agar plate and against nitrophenol-coproate and palmitate in liquid assays.

		W14	*E. coli* pBAD-*pdl*1 - AR	*E. coli* pBAD-*pdl*1 +AR
Tributyrin* [a]	(C=4)	-	-	-
Tween-20* [b]	(C=14<16)	++	+	+
Tween-80* [b]	(C=16<18)	++	-	-
p-nitrophenyl caproate**	(C=6)	+	-	-
p-nitrophenyl palmitate**	(C=16)	+	-	-

*= plate assays; - = no visible halo, lipase activity not detected;
+ = halos less than 15mm in diameter; ++ = halos greater than 15mm in diameter
[a] = lipase activity indicated by halo of clearance zone around colonies
[b] = lipase activity indicated by halo of opaque zone around colonies
**= liquid assays; - = OD_{410} below negative control (PBS);
+ = OD_{410} above negative control (PBS)

Table 5.3 Effects of whole cell lysate from Pdl1 expressing *E. coli* on mortality and phagocytic ability of primary *M. sexta* haemocytes in *in vitro* monolayer assays.

	PBS	*E. coli* pBAD-*pdl*1 - AR	*E. coli* pBAD-*pdl*1 +AR
Mortality [a]	3.1 ± 1.0	3.7 ± 0.6	4.1 ± 1.0
Phagocytosis [b]	86.8 ± 3.8	84.5 ± 6.2	80.5 ± 1.3

[a] = Trypan blue stained cells counted. Mean ± S.D. of >1500 cells counted from 10 random views per monolayer, one monolayer each of three larvae
[b] = Haemocytes co-localised with GFP-*E. coli* counted. Mean ±S.D. of >500 cells counted from 15 random view per monolayer, one monolayer each of three larvae

5.4 Discussion

Xenorhabdus and *Photorhabdus* strains have been observed to cause haemolysis on blood agar plates (Fischer-Le Saux *et al.*, 1999) and many of the newly emerging human isolates are strongly haemolytic (Peel *et al.*, 1999). However, the physiological significance of this phenomenon is still unclear as progress is hindered by the lack of understanding for how cloned haemolysins from these bacteria relate to the annular phenotype itself.

This chapter describes the cloning of a diacylglycerol lipase-like gene (*pdl*1) from *P. luminescens* W14 and shows that it encodes a protein that is haemolytic against sheep red blood cell both on blood plates and in liquid assays. Pdl1 expressed in *E. coli* accumulates in the cytoplasm and its ability to lyse red blood cells on agar plate is not dependent on the induction of the cryptic *E. coli* haemolysin SheA (Fernandez *et al.*, 1998). Both washed cells and whole cell lysate of Pdl1 expressing *E. coli* lyse sheep red blood cells in the liquid assay, suggesting possible membrane association also. Lipase assays performed against a range of artificial substrates show no detectable lipase activity associated to Pdl1. Further, Pdl1 containing whole cell lysate is not cytotoxic against *M. sexta* haemocytes, without any effects on viability or phagocytic competence.

Interestingly, *P. luminescens* W14 tested negative for haemolysis in the liquid assay while being capable of strong annular haemolysis on blood plate. These observations are consistent with the theory that annular haemolysis is a complex multi-factoral process. Additionally, gradients of counteractive or synergistic factors may be necessary to attain annular haemolysis, which is not possible in liquid media. Such synergistic haemolysis of erythrocytes is well documented in the CAMP effect (a combination of the initials of the authors who described the phenomenon) in which the haemolytic zones of *Actinobacillus pleuropneumoniae* are enlarged when grown in the vicinity of β-toxigenic *Staphylococcus aureus* (Frey *et al.*, 1989). In this case, *S. aureus* produces a phospholipase or sphingomyelinase which hydrolyses phospholipids from the erythrocyte membrane, making the erythrocytes more susceptible to haemolysis to haemolytic agents from *A. pleuropneumoniae* (Frey *et*

al., 1989). Therefore, although the recombinant *E. coli* expressing Pdl1 display a simple single clearance zone, it could be one of the haemolytic factors responsible for the complex annular haemolysis associated with the wild type bacterium.

The Pdl1 associated haemolysis appears to be intrinsic to the protein itself since its expression in a *sheA* mutant *E. coli* displayed haemolysis against sheep red blood cells on agar plate. In previous studies, the overproduction of heterologous regulators was able to induce a haemolytic phenotype caused by the silent haemolysin SheA (also referred to as ClyA, HlyE) encoded chromosomally in the *E. coli* laboratory strains used (Uhlich *et al.*, 1999; Fernandez *et al.*, 1998; del Castillo *et al.*, 1997). Recently, a *hol-1* locus in *X. nematophila* was described and suggested to aid the release of sheA in *E. coli* and speculated to participate in the release of various compounds (Brillard *et al.*, 2003). Pdl1 appears in the cytosol of *E. coli* and its secretion route is still obscure with the lack of signalling sequence at the locus, an antibody would be useful to track its presence in *Photorhabdus* and any association with haemocytes or other host tissues.

Whole cell lysate of Pdl1-expressing *E. coli* provides a source of Pdl1, liquid haemolysis assays with varied concentrations of Pdl1 and red blood cells were performed to deduce the haemolysis mode of action as described by Rowe and Welch (1994), however, the data obtained does not conclusively determine the modes of haemolysis. Further experiments are necessary which would be aided by the use of purified protein. Expression and purification of the protein and similar assays carried out should reveal information regarding its mode of action. It does remain possible that Pdl has both pore forming and enzymatic functions.

The predicted Pdl1 amino acid sequence shares homology with an *A. oryzae* lipase and the *V. cholera* hypothetical protein. All proteins compared by alignment bear the consensus pentapeptide sequence and the catalytic triad residues common in active lipases (Jaeger *et al.*, 1994). However, a variety of plate and spectrophotometric lipase assays with several substrates indicate a lack of lipase activity by the recombinant Pdl1. The positive results from wildtype *P. luminescens* W14 in some of the assays performed reveal the presence of other lipases. Further, the kinetics of lipases is complicated and highly dependent on substrate

concentration (Verger & de Haas, 1973) and substrate specificity and stereo-selectivity (Rogalska *et al.*, 1993). Therefore more thorough investigations are required to conclusively rule out lipase activity.

The homology shared between the *V. cholerae* open reading frame VC1418 and Pdl1 tempts speculation of a potential role for Pdl1 in the *P. luminescens* W14 anti-haemocytic phenotypes described in chapter 2. Many bacterial lipases have been characterised with specific anti-host immunity roles. *S. aureus* produces lipases that alter granulocyte surface structures and block phagocytic killing of bacterial cells (Rollof *et al.*, 1988) while *P. aeruginosa* lipase and phospholipase C inhibit monocyte chemotaxis (Jaeger *et al.*, 1991) and interferes with signalling in inflammatory processes (König *et al.*, 1994) disrupting cellular and humoral responses respectively. Interestingly, despite observed haemolysis against mammalian red blood cells, Pdl1 does not affect haemocyte viability or their ability to phagocytose in *M. sexta*. However, many bacterial virulence factors including lipases have been shown to work synergistically with other extracellular enzymes (Beecher & Wong, 2000). Jaeger *et al.* (1994) suggest that data from studies of single enzymes in isolation should be interpreted with care and may not necessarily explain the bacteria induced phenotypes.

The expression and purification of Pdl1 would be useful for structural and biochemical analysis. Its expression profile using either Western, Northern analysis or quantitative RT-PCR may shed light on potential roles in *Photorhabdus* virulence as demonstrated by Daborn *et al.* (2001). The *pdl1* locus is located in a pathogenicity island downstream of *tcdA1B1* with oral toxicity (Waterfield *et al.*, 2002), but oral bioassays where neonate *M. sexta* larvae were fed on artificial diet laced with Pdl1 expressing *E. coli* revealed the lack of affect on the larvae weight gain or viability (Dowling, 2000). Marokhazi *et al.* (2003) performed genomic DNA comparison between *Photorhabdus* isolates by hybridisation against a partial W14 microarray, it revealed *pdl1* to be present in seven of ten strains tested and some strains suggested to have more than one copy. In contrast *pdl2* may be present in three of the ten strains tested in lower copy numbers relative to *pdl1*, suggesting the better conserved Pdl1 is more likely to have important role.

The ultimate method to determine role in virulence is to knockout a single locus and monitor for changes in pathogenicity of the mutant relative to wildtype. Some efforts were spent in creating a *P. luminescens* W14 *pdl1* knockout using a negative selection plasmid. Two plasmid constructs were created containing the *B. subtilis* levansucrase gene *sacB*, an ampicillin resistant gene on the plasmid and the *pdl1*locus interrupted with either a streptomycin or kanamycin resistant gene. Unfortunately, these efforts were unsuccessful due to the high mutation-rate of the *sacB* selection gene in *P. luminescens* W14, which resulted in many false-positive clones. Furthermore, PCR was the only method to confirm knockout, a screen complicated by the presence of a second copy, *pdl2*, which was unknown at the time. Other methods described by Forst *et al.* (1997) should be explored to pursue this further. Because the presence of *pdl2* and numerous potential toxins may render Pdl1 functionally redundant and hence masking any reduction in pathogenicity of the *pld1*-knockout, all data should be cautiously interpreted.

This chapter describes the identification, cloning and expression of the *Photorhabdus* W14 *pdl1* and reveals its haemolytic phenotype against mammalian red blood cells. Although its function and potential role in virulence remain to be elucidated, the data available form encouraging basis for further research. Many known roles of lipid derivatives are important for cell functions such as cell structure / integrity, metabolic roles in enzyme activation, good source of energy in fatty acids and phospholipids in secondary messenger roles. Therefore Pdl1 could be essential for the bacteria's basic survival, however, with the above in mind, the lipase has numerous potential targets within host cells to disrupt targeted host cell functions as a virulence factor. Known lipolytic virulence factors have displayed a wide range of mode of action: attacks fatty-acid components in membrane of target host cells, interferes with fatty-acid components of signalling cascade, alters bacteria surface fatty-acids to prevent detection by host immune system, release of fatty-acids toxic to host. Also, degradative enzyme activities for biodegradation or bioconversion could deem Pdl1 as a symbiotic factor. Finally, given the presence of multiple *pdl*-homologues in the genome, it is possible that Pdl1 is active against a specific cell type or even a different species of insect.

Chapter 6

General discussion

Photorhabdus are entomopathogenic bacteria that lead a tripartite lifecycle with a symbiotic nematode partner, together forming a lethal complex against insect hosts (Forst *et al.*, 1997). *Photorhabdus* are potent pathogens with a LD_{50} at less then ten bacteria per insect (Forst *et al.*, 1997), however, the role of toxins in the pathogenicity of *Photorhabdus* is poorly understood (ffrench-Constant *et al.*, 2003; Dowds and Peters, 2002; Forst *et al.*, 1997). Haemocytes constitute the cellular components of the insect immune response that act in concert with humoral components (Tunaz *et al.*, 2003; Lavine and Strand, 2002; Trenczek, 1998). Haemocytes act to entrap invading microorganisms by phagocytosis and nodulation (Lavine and Strand, 2002; Stanley *et al.*, 1998; Hoffmann, 1995; Ratcliffe and Gagen, 1976). *Photorhabdus* must therefore have means to overcome or evade these immune responses within the insect host (Au *et al.*, 2004; ffrench-Constant *et al.*, 2003; Daborn *et al.*, 2002; Zumbihl *et al.*, 2002).

The complete genome sequence of *Drosophila* and its genetic malleability makes it the choice organism in molecular insect immunology research and a vigorous model system to elucidate mechanisms of vertebrate cell surveillance and immune gene regulation (Tzou *et al.*, 2002; Imler and Hoffmann, 2000; Medzhitov and Janeway, 1998b; Hoffmann *et al.*, 1996; Hultmark, 1993). However, the small size of the fruitflies is problematic for bacterial inoculation by injection and for blood cell collection. Lepidopteran larvae such as *G. mellonella* and *Spodoptera* spp. as well as *M. sexta* have been utilised as model host for *Xenorhabdus* and *Photorhabdus* pathogenicity research (Brillard *et al.*, 2003; Silva *et al.*, 2002; Han and Ehlers, 2000). Here the lepidopteran *M. sexta* is chosen for its large larvae for inoculation by injection and plentiful source of haemocytes for *in vitro* studies. This thesis describes the anti-haemocyte effects of soluble toxins of *P. luminescens* W14 in comparison to a non-orally toxic *P. temperata* K122 and a non-pathogenic *E. coli*, with the aims to test putative virulence factors and further the knowledge of anti-bacterial immune responses in *M. sexta*.

The effects of *P. luminescens* W14 soluble toxins against haemocyte were investigated using *in vitro* haemocyte monolayer assays. The data confirmed the previously observed W14 supernatant-induced haemocyte mortality and phagocytosis inhibition (ffrench-Constant *et al.*, 2003; Dean, 2002; Silva *et al.*, 2002). From the present studies, W14 supernatant was shown to contain toxin(s) that induced haemocyte mortality after 4 h, these toxin(s) which are absent from the supernatants of K122 and *E. coli*. However, K122 supernatant was shown to induce haemocyte mortality after a prolonged incubation of 24 h, hence toxins present in K122 supernatant are likely to have different modes of action. The toxic factors responsible for the W14-induced phenotypes observed were heat-liable (data not shown), however, the sensitive nature of haemocytes to their environments including *in vitro* chemical and enzyme treatments prevent further assessments of the biochemical nature of the soluable toxins. The attempts to characterise the W14-induced effects against more resilient immortalised *Drosophila* cell-lines; 1(2)mbn (Gateff *et al.*, 1980) and S2 (Invitrogen) were unsuccessful as no distinguishable phenotypes were observed (data not shown). Nevertheless, recent success in our laboratory in establishing a *Photorhabdus* infection in *Drosophila* adults (Gaelle Le Goff, 2003, unpublished) encourages further efforts to establish a *Photorhabdus* and *Drosophila* cell-line model for future investigations. Such model will enable *in vitro* assessment of *Photorhabdus*-haemocyte interactions with minimal variation that exists between primary haemocyte cultures. Furthermore, ultra-structural studies combined with the use of monoclonal anti-haemocyte antibodies would give insight into of the previously reported *Photorhabdus*-haemocytes interactions *in vitro* (ffrench-Constant *et al.*, 2003) as well as likely *in vivo* bacteria-haemocyte interactions.

Despite the characterised phenotypes of *P. luminescens* W14, the identity of the toxins responsible is unknown. Attempt to screen the W14 cosmid libraries, as well as testing candidate clones based on sequence homology to known toxins, have not led to positive identification of toxins (data not shown). Microarray technology would be useful in monitoring expression of *Photorhabdus* candidate genes either *in vitro* when exposed to haemocytes in comparison to Grace's insect media alone or during *in vivo* infection in comparison to *in vitro* growth. Work is currently underway in our laboratory.

P. luminescens W14 is the strain from which the orally toxic Toxin complex (Tc) toxins were originally isolated (Bowen and Ensign, 1998), this characteristic has since been found in other isolates (Marokhazi *et al.*, 2003). The *P. luminescens* W14 sample sequence analysis revealed multiple *tc* loci present in the W14 genome (Waterfield *et al.*, 2001b; ffrench-Constant *et al.*, 2000). Blackburn *et al.* (1998) showed TcA to cause larval gut epithelial cells death prior to insect death but alternate host cell targets of the Tc toxins have been speculated (ffrench-Constant *et al.*, 2003; Waterfield *et al.*, 2002b; Waterfield *et al.*, 2001b). Haemocyte toxicity of the Tc toxins were assessed in the present studies using *P. luminescens* W14 *tc*-knockout mutants and revealed Tca to have no observable contribution towards wildtype haemocyte toxicity. The absence of Tcb suppressed the W14 supernatant-induced inhibition of haemocyte phagocytosis of *E. coli*, however, the expression of Tcb alone in *E. coli* did not confer such supernatant-induced inhibition of phagocytosis. Further, granulocyte actin rearrangement was suppressed in the *tcb*-knockout mutant but the exogenously expressed Tcb did not show any observable changes in haemocyte actin cytoskeleton. Therefore W14 Tcb alone is not toxic, although knockout mutant studies indicate an important role in wildtype W14 supernatant toxicity.

Interestingly, assays with K122 expressing W14 *tc*s revealed disruption of the 24 h K122 supernatant toxicity, which implies likely interactions between TccC and Tcd. Waterfield *et al.* (2001) showed the co-expression of TccC with Tcd to be necessary to confer oral toxicity in *E. coli* but TccC is not required to confer oral toxicity in K122. Marokhazi *et al.* (2003) suggest Tcd to be linked to cell-associated oral toxicity and the present study showed that it interferes with K122 supernatant-induced haemocyte toxicity when over-expressed. These observations suggest TccC to have roles in W14 haemocyte and oral toxicity, perhaps a general role in secretion or protein modification which leads to toxin activation. The interaction between TccC and Tcd could be investigated further by over-expression of either locus in W14 to observe changes in W14 wildtype supernatant haemocyte toxicity as well as cell-associated oral toxicity. Large-scale expression and purification of Tc toxin components and combination of components *in vitro* in the search for active toxin complexes may help to identify toxins for haemocyte toxicity. Further, the effects of

Tcs on mammalian cells are being investigated in our laboratory and the ability to express individual loci and domains will aid the identification of toxin components.

Experimental data of *in vitro* and *in vivo* studies on W14 Mcf showed haemocyte toxicity, while the K122 Mcf was shown to have a role in pathogenicity, although it appears not to be necessary to cause insect death. Due to difficulties in producing gene knockouts in *P. luminescens* W14 as well as the presence in W14 of a close *mcf* homologue (Waterfield *et al.*, 2003), K122 was a useful tool to investigate the role of Mcf in *Photorhabdus* pathogenicity. Attempts made to study the expression profile of W14 Mcf using Western analysis were unsuccessful, this was possibly due to high limit of detection for the available antibodies for the concentration of toxin present. The more sensitive method of quantitative RT-PCR was used in attempt to trace the expression profile of K122 *in vivo*, however, technical problems were not overcome within time available to obtain conclusive results (data not shown). Both W14 Mcf1 and Mcf2 are currently being investigated in order to locate the functional domain and to elucidate their mode of action using mammalian cell lines (Dowling *et al.*, 2004).

The *pdl1* locus was isolated from a high-throughput screen of a *P. luminescens* W14 plasmid library (previous unpublished data). *In vitro* assays revealed a lack of lipase activity but Pdl1 displayed haemolytic properties both on agar plate and in liquid assays, which may contribute towards the annular haemolysis phenotype of wildtype W14 on agar plate. Interestingly, wildtype *P. luminescens* W14 showed negative results in the liquid haemolysis assay, hence the mechanism of annular haemolysis remains unclear but is likely to involve multiple factors that work in synergy, as shown in previously documented cases (Beecher and Wong, 2000; Jansen *et al.*, 1995). The mode of blood cell lysis in also unclear as data from liquid assays with varying concentrations of Pdl1 and blood cells suggest contradictory conclusions. Like Mcf, there is a close homologue present within the W14 genome and in other isolates suggesting the gene product has a role that is advantageous to the pathogen. *E. coli*-expressed Pdl1 did not show toxicity towards *M. sexta* haemocytes, the expression profile would advance identifying the role of Pdl1 in *P. luminescens* W14.

In summary, the present study characterised the *P. luminescens* W14 supernatant-induced phenotypes of *M. sexta* haemocytes. Data suggest W14 and K122 to have distinct virulence mechanism to suppress haemocyte phagocytosis while non-pathogenic *E. coli* is susceptible. Several toxins were investigated for their contribution towards these phenotypes; here I report supporting data for *P. luminescens* W14 Tcb, TccC, Tcd and *P. temperata* K122 Mcf to have roles in pathogenicity of *Photorhabdus* in a *M. sexta* infections. The traditional method to confirm toxin activity is to knockout the locus and to observe the loss or attenuation of virulence (as measured by the in LD_{50} value). However, as the *P. luminescens* W14 genome contains numerous putative virulence factors by sequence homology and many in multiple copies, the lose of any single locus is likely to cause much more subtle changes in virulence in LT_{50} (as seen in the K122 *mcf* knockout). Therefore an understanding of the 'normal' immune responses of *M. sexta* to bacterial infection is important.

Insect immunity has been studied for over a century and many insects have been explored as model hosts for bacterial infection research (Steinert *et al.*, 2003; Couillault and Ewbank, 2002; Jander *et al.*, 2000). The humoral factors in insect immunity are already studied at molecular level (Boman, 2003; Tzou *et al.*, 2002; Williams, 2001; Otvos, 2000; Gillespie *et al.*, 1997; Ashida, 1990) while progress in understanding the cellular responses has been hindered by many problems and have therefore received less attention (Lavine and Strand, 2002; Ramet *et al.*, 2002; Gillespie *et al.*, 1997; Ratcliffe, 1993). Haemocyte count fluctuations were reported in response to bacterial infection in *M. sexta* larvae (Dunn and Drake, 1983; Horohov and Dunn, 1982), the present study has confirmed those observations and has shown that haemocyte count are depleted without a following increase during *Photorhabdus* infections. However, the rapid depletion of haemocyte count appeared not to influence the timing of insect death as suggested by the delayed larval death in K122 *mcf* knockout mutant that displayed similar rate of haemocyte count decline relative to that in wildtype K122 infected larvae.

Additionally, haemocyte count fluctuations of *M. sexta* larvae injected with varying doses of *E. coli* showed *M. sexta* have a great capacity for phagocytosis, removing about 9.9×10^7 colony forming bacteria within 2 h post injection. However, the level

of phagocytosis appeared to depend on the initial number of bacterial injected, i.e. fewer were removed within the same time period in larvae injected with lower number of bacteria. Further, the haemocyte count profile varied with the initial number of bacteria injected, and a second peak of haemocyte count was observed in insects injected with the highest number of bacteria, implying a possible 'threshold' of viable bacterial count in operation which promotes haemocyte proliferation and / or release from sessile state once this threshold is exceeded. Several humoral molecules, the eicosanoid biosynthesis pathway as well as the phenoloxidase signalling pathway have been shown to influence cellular immune responses (Tunaz *et al.*, 2003; Yu and Kanost, 2003; Yu *et al.*, 2002; Stanley *et al.*, 1998; Kanost *et al.*, 1994; Miller *et al.*, 1994). The use of *Drosophila* cDNA microarrays is currently underway in our laboratory using the S2 *Drosophila* cell line exposed to either *E. coli* or *P. luminescens* W14 to monitor the mRNA profile of the cells in response to the bacteria using a similar protocol to that performed by De Gregorio *et al.* (2001).

Further insight into the importance of particular molecules involved in regulation of haemocyte proliferation would be valuable. However, it should be noted that the haemocyte count recorded in this present work was that of circulating haemocytes, which does not account for sessile haemocytes, so that changes in haemocyte count could simply mean increased or decrease haemocyte adhesiveness within the insect. It is currently difficult to compare these findings with others in literature due to the use of different host and bacteria combinations (Ratcliffe, 1993).

Insect immunity is often compared to the vertebrate innate immune system, which provides rapid and non-specific responses to microbial infections that are independent of previous exposure (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 1997). However, insects lack the adaptive elements including specific antibodies and immune 'memory' which allows more efficient of secondary infections (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 1998a). Nevertheless, insects have been reported to enter a 'primed' state for a period post infection, where the immune system become immuned to an infection that is otherwise lethal in naive individuals (Mulnix and Dunn, 1995). This is thought to coincide with the third phase of immune response as described by Ratcliffe (1993) where humoral anti-microbial peptides would be present in abundance and hence

able to mount a more efficient response to combat an infection compared to naive insects. Indeed, attenuation of *Photorhabdus* pathogenicity has been shown in our laboratory in larvae previously injected with *E. coli* (J. Marokhazi and I. Eletherianos, personal communication). Research is currently underway to follow up this recent observation. By using similar haemocyte count profiling methods, available antibodies specific to haemocyte types and anti-microbial peptides to correlate the occurrence of various components timing of the 'primed' state against *Photorhabdus* infections, it may be possible to identify anti-*Photorhabdus* immune effector(s). Interestingly, the recently reported hyperphagocytes (HPs) by Dean (2002) were only observed in *M. sexta* larvae injected with 1×10^7 or greater number of *E. coli* per *M. sexta* larva (data not shown). Moreover, the present study showed the HPs to be resistant to *P. luminescens* W14 supernatant-induced inhibition in phagocytosis, hence HPs could be the components in the 'primed' larval state that are capable of removing *Photorhabdus* from the larval haemocoel. Screening for a HP-specific monoclonal antibody would be useful for future studies. The observations presently reported on the anti-bacterial immune responses in *M. sexta* against *E. coli* and further studies using such *Photorhabdus-M. sexta* models will undoubtedly add to the state of knowledge in insect immunity.

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Cover illustration: *Photorhabdus* is an insect pathogenic bacterium delivered into the insect blood system (hemocoel) via its nematode carrier. Once inside the hemocoel it meets the insect phagocytes, termed 'hemocytes'. Normally the hemocytes engulf foreign bacteria by phagocytosis, however *Photorhabdus luminescens* W14 secretes factors into the bacterial supernatant which inhibit phagocytosis. The cover image shows the actin cytoskeletons of one type of hemocyte, plasmatocytes, after treatment with W14 supernatant. The strongly FITC phalloidin staining bundles of actin within the treated cells resemble stress fibres and suggest that factors in the W14 supernatant can reorganise the cytoskeleton, thereby inhibiting phagocytosis. For further details, readers are referred to the article by Au *et al.* on pp. 89 of this issue.

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Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes

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Summary

***Photorhabdus* are insect pathogenic bacteria that replicate within the insect haemocoel following release from their entomopathogenic nematode symbionts. To investigate how they escape the cellular immune response we examined the effects of two strains of *Photorhabdus*, W14 and K122, on *Manduca sexta* phagocytes (haemocytes), *in vitro* and *in vivo*. Following injection of *Esherichia coli* into *Manduca* larvae, these non-pathogenic bacteria are rapidly cleared from the haemolymph and the number of free haemocytes transiently increases. In contrast, following injection of either strain of pathogenic *Photorhabdus*, the bacteria grow rapidly while the number of haemocytes decreases dramatically. *In vitro* incubation of haemocytes with either *Photorhabdus* supernatant reduced haemocyte viability, and the W14 supernatant caused distinct changes in the actin cytoskeleton morphology of different haemocyte cell types. In phagocytosis assays both *Photorhabdus* strains can inhibit their own phagocytosis whether the bacterial cells are alive or dead. Further, the supernatant of W14 also contains a factor capable of inhibiting the phagocytosis of labelled *E. coli*. Together these results suggest that *Photorhabdus* evades the cellular immune response by killing haemocytes and suppressing phagocytosis by mechanisms that differ between strains.**

Introduction

Photorhabdus and *Xenorhabdus* are insect pathogenic bacteria vectored by entomopathogenic nematodes (Forst and Clarke, 2002; ffrench-Constant *et al.*, 2003). Following penetration of the nematode into the insect the bacteria are regurgitated directly into the haemocoel of the insect host (Ciche and Ensign, 2003). Subsequently the bacteria grow unrestricted by the insect immune system (Daborn *et al.*, 2001), releasing toxins to kill the insect host (ffrench-Constant *et al.*, 2003) and also serving as a food source for their nematode symbionts (Forst and Clarke, 2002). In contrast, when infected by non-pathogenic *Esherichia coli*, insects can normally clear the bacteria via a combination of phagocytosis, nodulation (binding of multiple haemocytes to aggregations of bacteria) and the release of antibacterial peptides (Lavine and Strand, 2002). Given that *Photorhabdus* can evade the insect immune system this suggests that it may either destroy insect haemocytes or inhibit their phagocytic ability.

Phagocytosis is a membrane-driven process driven by the host cell actin cytoskeleton that results in the uptake of invading bacteria (Castellano *et al.*, 2001). This is a conserved process associated with reorganization of the actin cytoskeleton by Rho family GTPases (Etienne-Manneville and Hall, 2002). In mammals phagocytosis is facilitated by association of the particle to be engulfed with various opsonins, such as constant portions of immunoglobulins, FcR, or complement receptor, CR3 (Castellano *et al.*, 2001) or non-opsonic pattern cell surface recognition molecules such as mannose receptors (Stahl and Ezekowitz, 1998). Bacteria have evolved numerous toxins and type III-delivered effector molecules which can interfere with the actin cytoskeleton and inhibit phagocytosis (Ernst, 2000; Barbieri *et al.*, 2002). Although well-documented in vertebrate immunity, it remains unclear how insect pathogens evade phagocytosis. In Lepidoptera (butterflies and moths) two types of haemocytes, granulocytes and plasmatocytes, have been reported to be phagocytic, although their relative contributions vary between insect species (Lavine and Strand, 2002). However the mechanisms whereby *Photorhabdus* avoids phagocytosis via these two groups of cells are undocumented.

Previous work using haemocyte monolayers from *Spodoptera littoralis* has shown two distinct haemolytic activities in supernatants from cultures of *Xenorhabdus nematophila* (Brillard *et al.*, 2001). The first cytolytic activity appears when bacterial growth enters stationary phase and is active against both sheep red blood cells and insect granulocytes. The second peak of activity occurs later in stationary phase, causing haemolysis of rabbit red blood

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cells and insect plasmatocytes. Insertional activation of the bacterial *fhlD* gene led to loss of the first activity while preserving the second, suggesting that they are under independent genetic control (Brillard *et al.*, 2001). The same study also suggested that there was no haemolytic activity associated with the supernatants of various *Photorhabdus* strains. Previous work on *Photorhabdus* strain W14, however, has shown that the bacterial supernatant can suppress the phagocytosis of *E. coli* in monolayer assays (Silva *et al.*, 2002). In order to clarify the effects of different *Photorhabdus* strains here we investigate the ability of two strains W14 and K122 (corresponding to two different species *P. luminescens* ssp. *akhurstii* and *P. temperata* ssp. *temperata*, respectively) to persist within the lepidopteran model host *Manduca sexta*. We examine the number, viability and phagocytic competence of haemocytes present during a *Photorhabdus* infection and examine changes in their cellular morphology when treated with *Photorhabdus* supernatants.

Results

Survival of Photorhabdus *and* E. coli *within infected* Manduca

In order to document how non-pathogenic *E. coli* and two different pathogenic *Photorhabdus* strains (W14 and K122) survive in *M. sexta*, we injected bacteria into a cohort of insects and then recorded the numbers of recoverable bacteria over time. Following injection of 10^5 *E. coli* into larval *Manduca*, the bacteria are rapidly cleared from the haemolymph with none being recoverable after 36 h (Fig. 1A). At the same time, the number of free haemocytes increases by 8 h, before returning to the resting level of 2×10^6 per insect (Fig. 1B). In contrast, following injection of 100 cells of either *Photorhabdus* W14 or K122 the number of recoverable bacteria increases exponentially reaching a peak of 10^8 recoverable CFU after 60 h (Fig. 1A) whilst the number of free haemocytes declines over time with few haemocytes being recoverable at 60 h (Fig. 1B). With *Photorhabdus* strain K122 this decline in haemocytes is immediate, whereas following injection of strain W14 the number of haemocytes falls after 8 h and declines steadily until the time of insect death.

Survival and morphology of haemocytes treated in vitro

Given the rapid decline in haemocyte numbers associated with *Photorhabdus* infection we investigated the toxicity of *Photorhabdus* supernatants to haemocytes *in vitro*. Following incubation of haemocytes with either PP3 media alone or cell-free *E. coli* supernatant, little reduction in haemocyte viability was recorded over 24 h (Fig. 2). However, following incubation of haemocytes with supernatants from cultures of either *Photorhabdus* W14 or K122 there was a rapid reduction in haemocyte viability with 25%, or fewer, haemocytes still viable after 24 h. Confocal microscopy of haemocytes stained with TRITC-phalloidin showed dramatic changes in actin cytoskeleton morphology following treatment with W14 supernatant (Fig. 3). Untreated granulocytes show a marked polarization in

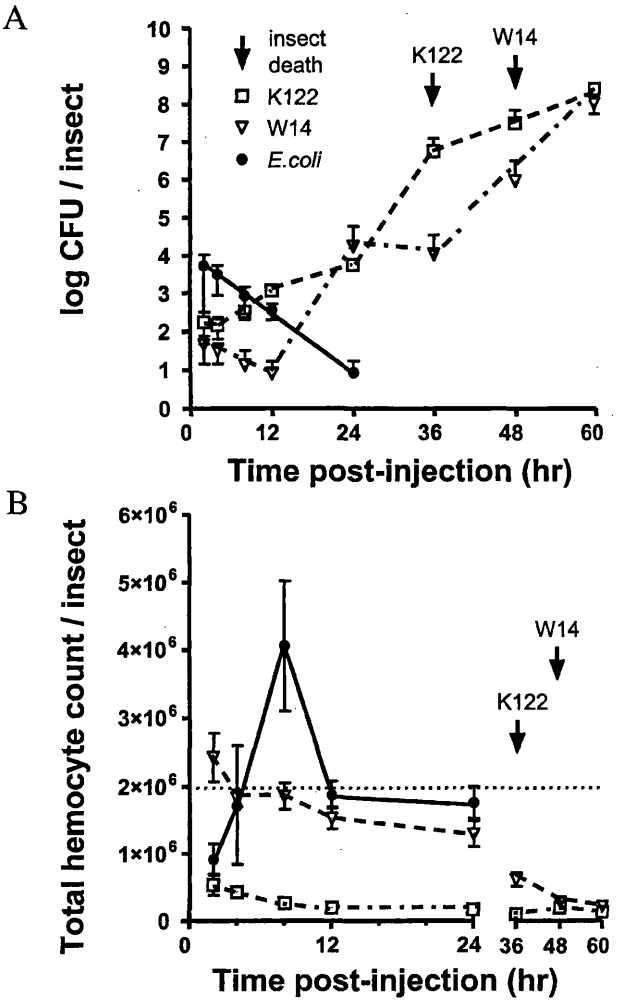

Fig. 1. Numbers of bacteria and haemocytes during *Photorhabdus* infection of *Manduca*.
A. Numbers of recoverable bacteria (CFU, colony forming units) over time following injection of *Manduca* larvae with *E. coli*, *Photorhabdus* strain W14 or *Photorhabdus* K122. Larvae were injected with bacteria and then bled at different time intervals to determine the number of recoverable CFU in the insect haemolymph. Note that *E. coli* are cleared within 36 h, whereas both *Photorhabdus* strains grow rapidly over time.
B. Estimated total free haemocyte count over time following injection of *E. coli*, *Photorhabdus* W14 or K122. Larvae were injected with bacteria and then bled at different time intervals to determine the number of free haemocytes. Note the transient increase in free haemocytes after *E. coli* injection and the decline in haemocytes following injection of either *Photorhabdus* strain. Arrows indicate the average time of insect death observed with each *Photorhabdus* strain.

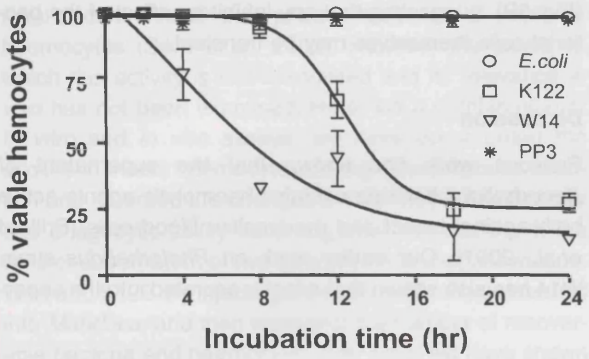

Fig. 2. Viability of haemocytes treated with bacterial supernatants. Viability of haemocytes treated with either PP3 broth or supernatant from *E. coli*, *Photorhabdus* W14 or K122. Caterpillars were bled onto a coverslip to make a haemocyte monolayer. Haemocyte monolayers were then exposed to the different treatments and haemocyte viability recorded over time via live-dead staining. Note the rapid decline in viability following treatment with either *Photorhabdus* supernatant.

actin staining with intense staining adjacent to the probable leading edge of the cell. This morphology is not affected by treatment with *E. coli* or *Photorhabdus* K122 supernatants, but following treatment with W14 supernatant actin polarization is lost and treated cells appear shrunken with marked filopodia (microspikes). Untreated plasmatocytes appear well spread and show a diffuse actin cytoskeleton which appears unchanged following treatment with *E. coli* or *Photorhabdus* K122. Following a 4 h treatment with *Photorhabdus* W14 supernatant plasmatocytes show two dramatic changes in morphology. First, and most commonly, they show the presence of severe actin bundling (stress fibres). Second, in some cases, the cells appear to have been destroyed leaving only punctate pattern of staining (Fig. 3). We interpret this to be remnants of actin-rich points of contact between the destroyed cell and the substrate.

Haemocyte phagocytosis in vitro *and* vivo

Finally, to investigate the effect of these changes in morphology and viability on the phagocytic competence of the haemocytes we examined their ability to phagocytose either *Photorhabdus* or *E. coli*, *in vitro* or *vivo*. *In vitro*, phagocytosis of cells of either strain of *Photorhabdus* is reduced compared to *E. coli* controls. This effect is still apparent whether the *Photorhabdus* cells are alive or dead (Fig. 4A). However, live W14 cells show a significantly stronger inhibition than the other treatments (Fig. 4A). This suggests that cells of both K122 and W14 may inhibit their own phagocytosis and that, further, live W14 bacteria may also secrete an additional non-cell-associated anti-phagocytic factor. To test this hypothesis we looked at the effect of bacterial supernatants alone on the phagocytosis of labelled *E. coli*. Both the cells and supernatant of W14 were able to inhibit the phagocytosis

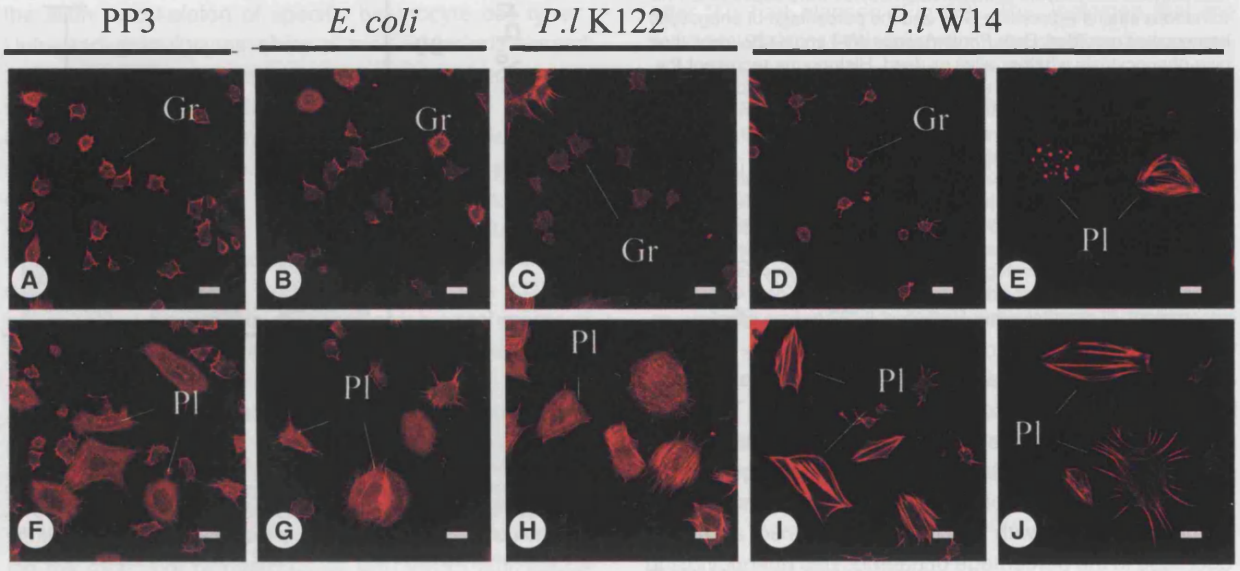

Fig. 3. Actin morphology of haemocytes treated with bacterial supernatants.
Haemocyte monolayers were treated with PP3 broth, *E. coli*, *Photorhabdus* K122 or *Photorhabdus* W14. Treated monolayers were then stained with FITC phalloidin to visualize their actin cytoskeletons. Typical staining patterns for two different cell types, granulocytes (Gr) and plasmatocytes (Pl), are shown. Note the polarized actin staining in the PP3 (A), *E. coli* (B) and *Photorhabdus* K122 (C) treated granulocytes, which disappears following treatment with W14 supernatant and is replaced by the presence of microspikes (D). Note also the induction of actin rich stress fibres in *Photorhabdus* treated plasmatocytes (E, I and J).

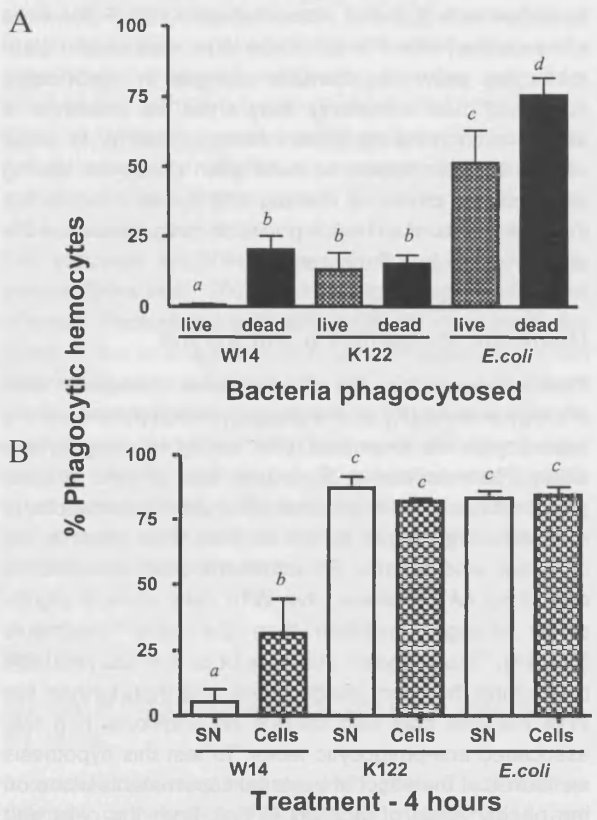

Fig. 4. Phagocytosis of *E. coli* or *Photorhabdus* by *Manduca* haemocytes *in vitro*.
A. Phagocytosis of GFP expressing *Photorhabdus* by insect haemocytes. Monolayers of insect haemocytes were exposed to *Photorhabdus* strains expressing GFP and the percentage of phagocytic haemocytes recorded. Both *Photorhabdus* W14 and K122 inhibit their own phagocytosis whether alive or dead. Histograms represent the mean of three experiments and their associated standard errors. Means carrying the same letter are not statistically different at the 5% level (paired *t*-test). Note the significantly greater effect of the live W14 bacteria over the other *Photorhabdus* treatments.
B. Phagocytosis of FITC labelled *E. coli* after treatment with different *Photorhabdus* strains and fractions. Monolayers of insect haemocytes were exposed to different *Photorhabdus* strains and fractions (cells and supernatant) and their ability to phagocytose FITC labelled *E. coli* recorded. The percentage of haemocytes phagocytosing *E. coli* is reduced by addition of either *Photorhabdus* W14 cells or 10% supernatant. In contrast either fraction of K122 has no effect.

of labelled *E. coli* whereas similar treatments from *Photorhabdus* K122 and *E. coli* could not (Fig. 4B). This confirms the hypothesis that W14 secretes an additional anti-phagocytic factor into its supernatant that is lacking from strain K122. *In vivo*, coinjection of W14 supernatant into *Manduca* injected with *E. coli*-GFP produced a marked decrease in the percentage of haemocytes phagocytosing bacteria (Fig. 5A), whereas coinjection of supernatants from either K122 or *E. coli* had no effect. Finally, injection of washed cells of either W14 or K122 strains into *Manduca* did not alter the percentage of phagocytes that subsequently (after 2 h) internalized injected *E. coli*-GFP (Fig. 5B), suggesting that any inhibitory effect of the bacterial cells themselves may be transient.

Discussion

Previous work has shown that the supernatant of *Xenorhabdus* bacteria contains haemolytic agents active both against insect and mammalian blood cells (Brillard *et al.*, 2001). Our earlier work on *Photorhabdus* strain W14 has also shown that a factor secreted into the super-

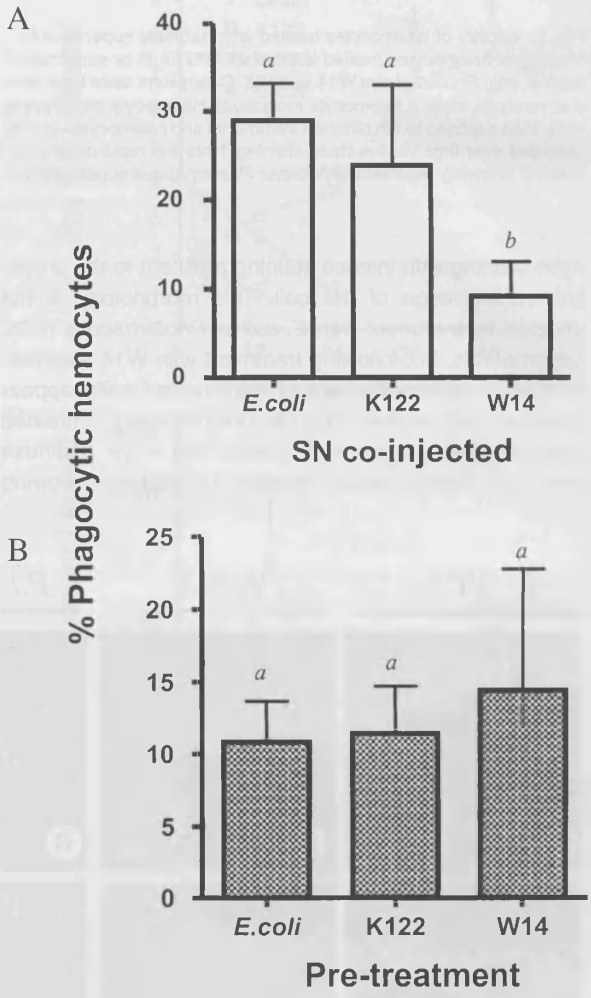

Fig. 5. Phagocytosis of *E. coli* GFP in *Manduca in vivo*.
A. Co-injection of *E. coli*-GFP and different bacterial supernatants into *Manduca* larvae to study the effects on phagocytosis *in vivo*. Co-injection of W14 supernatant into *Manduca* alongside *E. coli*-GFP inhibits the phagocyosis of *E. coli*-GFP. Histograms represent the mean of three experiments and their associated standard errors. Means carrying the same letter are not statistically different at the 5% level (paired *t*-test).
B. Pre-treatment of *Manduca* larvae with different bacterial cells to study the effects on phagocytosis *in vivo*. Injection of either *Photorhabdus* W14 or K122 cells followed by injection of *E. coli*-GFP has no effect on the subsequent rate of phagocytosis.

natant can inhibit the phagocytosis of *E. coli* by haemocytes (Silva *et al.*, 2002). However the extent to which this activity is cell-associated and its relevance *in vivo* has not been examined. Here, via a combination of *in vitro* and *in vivo* assays, we have documented the decline in insect haemocytes during *Photorhabdus* infection and recorded the changes in haemocyte morphology and phagocytic ability occurring after treatment with the cells or supernatant of two different *Photorhabdus* strains, W14 and K122. By injecting either *E. coli* or *Photorhabdus* into *Manduca*, and then recording the number of recoverable bacteria and haemocytes over time, we have shown a dramatic difference in the patterns of infection. After *E. coli* injection all the bacteria are rapidly cleared, as previously documented (Dunn and Drake, 1983); this period of clearance is also associated with an increase in the number of recoverable haemocytes, suggesting that exposure to the bacteria leads to the appearance of more phagocytes free in the insect haemocoel. In contrast, both strains of *Photorhabdus* grow unhindered within injected *Manduca* while the number of free haemocytes decreases dramatically. Thus at 60 h post injection a caterpillar may contain 10^8 *Photorhabdus* and virtually no viable haemocytes.

To investigate the hypothesis that the supernatants of either strain contain agents capable of killing haemocytes, we monitored haemocyte survival over time following *in vitro* treatment with bacterial supernatants. *Photorhabdus* W14 supernatants caused marked decreases in cell viability and also marked changes in the actin cytoskeleton of specific haemocyte cell types. Untreated granulocytes show a marked polarization of their actin cytoskeletons with a thickening of actin, presumably associated with the leading edge of the cell. Following supernatant treatment, this polarity disappears and the cell bodies appear contracted with the presence of numerous filopodia or microspikes. Plasmatocytes, which normally appear well spread with a diffuse actin staining, show marked stress fibre formation or, following destruction of the cell, a pattern of punctate staining highlighting the remnants of the actin-rich attachments of the cell to the substrate. Numerous bacterial toxins reorganize the actin cytoskeleton of target cells (Barbieri *et al.*, 2002) but in this case we would like to highlight two points. First, stress fibre formation can be caused by activation of the Rho GTP binding proteins (Etienne-Manneville and Hall, 2002). Second, a pattern of staining similar to the punctate pattern has been observed following the treatment of haemocytes with the *Photorhabdus* toxin Mcf. This toxin promotes apoptosis in both haemocytes and cells of the midgut epithelium (Daborn *et al.*, 2002). However, the presence of Mcf in the *Photorhabdus* supernatant has not been documented and is therefore under investigation.

To examine the effects of these morphological changes on phagocytic competence we examined the ability of haemocytes treated *in vitro* to phagocytose either *Photorhabdus* or *E. coli* expressing GFP. Both strains of *Photorhabdus* can inhibit their own phagocytosis whether alive or dead (Fig. 4A). However the inhibitory effect produced by live W14 bacteria is significantly greater, suggesting that W14 may also secrete an additional factor responsible for inhibiting phagocytosis. This assumption is validated by the observation that both the supernatant and the cells of W14 can inhibit the phagocytosis of labelled *E. coli* (Fig. 4B) whereas neither preparation from K122 can. Together these results show that both strains of *Photorhabdus* can inhibit their own phagocytosis by insect haemocytes but that the mechanisms differ between strains. Thus cell-associated factors are present in both K122 and W14 but only strain W14 carries an antiphagocytic factor in its supernatant. The molecular basis of phagocytosis inhibition remains undetermined but the current sequencing of two *Photorhabdus* genomes should provide candidate loci for genetic dissection.

To examine the effect of *Photorhabdus* cells and supernatants *in vivo* we examined the ability of these two different fractions to interfere with phagocytosis of concurrently injected *E. coli* expressing GFP. Again the results were different for each strain, with only W14 supernatants producing a marked reduction in the percentage of haemocytes containing *E. coli* (Fig. 5A). In contrast, injection of either W14 or K122 cells into caterpillars did not inhibit the ability of haemocytes to take up *E. coli*-GFP after 2 h had elapsed (Fig. 5B). This indicates that the adverse effect of *Photorhabdus* cells on *E. coli* phagocytosis may have a limited life within the insect itself. In conclusion this suggests that both types of *Photorhabdus* cells are able to inhibit their own phagocytosis but that K122 still permits treated haemocytes to phagocytose *E. coli*, whereas the greater cytotoxicity of W14 does not. Presumably in the course of a natural *Photorhabdus* infection, *Photorhabdus* needs to inhibit its own phagocytosis by haemocytes but perhaps initially not to alter their ability to uptake potential bacterial competitors entering the infected insect. As colonization of the infected insect by soil-associated bacteria is likely following nematode penetration, strains like K122 may enjoy reduced bacterial competition by not rendering insect haemocytes useless against other invaders while still inhibiting their own phagocytosis.

Experimental procedures

Insects and bacterial strains

Manduca sexta were reared as described elsewhere (Reynolds and Nottingham, 1985), newly moulted fifth instar larvae were used for all experiments in this study. The bacterial strains used

were *E. coli* XL1-Blue, *P. luminescens* ssp. *akhurstii* strain W14 (Bowen *et al.*, 1998), *P. temperata* ssp. *temperata* strain K122 (Wang and Dowds, 1993) and *E. coli*-GFP [Kanamycin resistant (Kn) used at 50 μg ml^{-1}]. Bacterial cultures were routinely cultured at 30°C with constant aeration in Luria-Bertani (LB) broth or on LB agar, except *Photorhabdus* W14 which was cultured in 2% proteose peptone No.3 (PP3) broth or on PP3 agar. Supernatant (SN) was obtained from stationary phase cultures 48 h post inoculation with fresh plate colonies, cells were removed by centrifugation and filter-sterilised. Bacterial cells were harvested from exponential phase cultures, washed three times with PBS and resuspended in the appropriate media. Cell counts were estimated by optical density at 600 nm and serial dilutions previously calibrated to a known number of colony forming units (CFU).

Total haemocyte count and colony forming units in vivo

Fifth instar *M. sexta* were chilled on ice for 15 min, surfaced sterilized with 70% ethanol and injected with 10 μl Grace's Insect Medium (GIM) containing either *E. coli* (1×10^5), W14 or K122 (100) directly into the haemocoel using a 100 μl Hamilton syringe with a 30-gauge needle. At fixed time-points over a period of 60 h post injection, the insects were chilled on ice, cut at the midpoint of the dorsal horn and bled into a prechilled sterile polypropylene tube. Aliquots of haemolymph (50 μl) were immediately added to prechilled GIM (450 μl). The total haemocyte count was determined microscopically at 100 × with a haemocytometer, the average of three counts per insect of six insects per time-point was calculated. Serial dilutions of the haemolymph were plated onto appropriate nutrient agar and CFU recorded 48 h later, the average of six insects per time point per treatment was recorded. The THC and CFU per insect were calculated assuming 320 μl of haemolymph per insect (haemolymph volume of newly moulted fifth instar larvae was 320 ± 84 μl, $n = 10$) and 50 μl per insect after death (haemolymph volume of infected larvae post death was 48 ± 18 μl, $n = 10$).

Haemocyte viability and actin morphology

Fifth instar larvae were chilled, surface sterilized and bled into prechilled GIM and cell density was adjusted accordingly to 5×10^6 ml^{-1}. Haemocyte suspensions (100 μl) were seeded onto each coverslip (10 mm in diameter) and left for 1 h at room temperature (RT) to allow haemocytes to attach. The monolayers were washed three times with GIM then incubated in a single well of a 24-well plate (Nunc) in 360 μl of GIM with an added 40 μl (10%) of treatment and kept at 28°C. At designated time points over 24 h, monolayers were removed from the wells, washed three times with GIM and trypan blue (0.02% in PBS) stained for 10 min at RT, washed three times with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS. Trypan blue stained and total cell counts were visualized and recorded with light microscope at 200× magnification. For fluorescein-conjugated phalloidin labelling, the PFA treated coverslips were incubated in a fresh solution of ammonium chloride (13.3 mg ml^{-1} in PBS) 10 min at room temperature, washed three times in PBS. Then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, washed three times in PBS and inverted onto 60 μl each of TRITC-phalloidin (Sigma) for 30 min at room temperature in darkness. These were washed three times in PBS, three times in distilled water and then mounted onto slides with 5 μl of mowiol mounting reagent per coverslip. Labelled haemocytes were visualized using a Zeiss LSM-510 confocal microscope.

Phagocytosis assays

The ability of insect haemocytes to phagocytose both *E. coli* and *Photorhabdus* itself was investigated. Assays involving *E. coli* used dead FITC labelled *E. coli* cells whereas assays involving *Photorhabdus* used GFP labelled bacteria. GFP labelling of *Photorhabdus* allowed us to look at the effect of live as well as dead bacteria. Haemocyte monolayers for *in vitro* assays were prepared as described above, 40 μl of *E. coli*-GFP (1×10^7 ml^{-1}) was added to each well 15 min after addition of each treatment, then left undisturbed for 4 h at 28°C. Dead bacteria used in phagocytosis assay were incubated in 4% PFA for 30 min at room temperature, then washed three times in PBS and cell suspensions were streaked onto appropriate agar plates to check for complete killing. For *in vivo* co-injection assays, fifth instar *Manduca* larvae were injected, as described above, each larvae was injected with 10 μl of PP3 or bacterial supernatant containing 1×10^7 *E. coli*-GFP and incubated at 25°C for 2 h. Larvae were then chilled, bled and monolayers prepared and stained as above. *In vivo* pretreatment involved injecting larvae with 10 μl of GIM containing 1×10^5 of either *E. coli*, W14 or K122; after 2 h at 25°C a second injection of 1×10^7 *E. coli*-GFP in 10 μl of GIM was administered and the larvae were incubated at 25°C. The larvae were chilled, bled and monolayers prepared and stained after a further 2 h. The monolayers were visualized under confocal microscope and percentage of phagocytic haemocytes determined by counting TRITC-phalloidin stained haemocytes co-localized with the *E. coli*-GFP in relation to total haemocyte count. Data shown were calculated from counts of 300–600 cells/monolayer/insect and three insects per treatment.

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We thank all in the laboratory of D. Clarke at Bath for useful discussions. C.A. was supported by a PhD studentship from the BBSRC and work in the ff.-C. and S.E.R. laboratories is supported by a grant from the Exploiting Genomics Initiative from the BBSRC.

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A single *Photorhabdus* gene, *makes caterpillars floppy (mcf)*, allows *Escherichia coli* to persist within and kill insects

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***Photorhabdus luminescens*, a bacterium with alternate pathogenic and symbiotic phases of its lifestyle, represents a source of novel genes associated with both virulence and symbiosis. This entomopathogen lives in a "symbiosis of pathogens" with nematodes that invade insects. Thus the bacteria are symbiotic with entomopathogenic nematodes but become pathogenic on release from the nematode into the insect blood system. Within the insect, the bacteria need to both avoid the peptide- and cellular- (hemocyte) mediated immune response and also to kill the host, which then acts as a reservoir for bacterial and nematode reproduction. However, the mechanisms whereby *Photorhabdus* evades the insect immune system and kills the host are unclear. Here we show that a single large *Photorhabdus* gene, *makes caterpillars floppy (mcf)*, is sufficient to allow *Escherichia coli* both to persist within and kill an insect. The predicted high molecular weight Mcf toxin has little similarity to other known protein sequences but carries a BH3 domain and triggers apoptosis in both insect hemocytes and the midgut epithelium.**

insecticide | *Photorhabdus* | pathogenicity | apoptosis | toxin

Characterization of bacterial genomes involved in pathogenicity and symbiosis is important to define the patterns of gene acquisition or loss involved in the evolution of these traits (1–4). The bacterium *Photorhabdus luminescens* forms a model system in this context as it has both symbiotic and pathogenic phases of its lifecycle (5, 6). *P. luminescens* is also a member of the Enterobacteriaceae, facilitating comparison of putative virulence/symbiosis factors with well studied bacteria such as *Escherichia coli* (7). Previous genomic sample sequencing of *P. luminescens* subsp. *akhurstii* strain W14 identified numerous genes with homology either to known virulence factors from *P. luminescens*, such as the *toxin complex* (*tc*) genes (8) or to putative virulence factors identified in other pathogenic bacteria (7). However, 53% of the genes sampled are clearly distinct from those found in the genome of *E. coli* K12 (7), suggesting that the *P. luminescens* genome may contain a large number of novel genes involved either in pathogenicity, symbiosis, or both.

Photorhabdus luminescens has an unusual lifecycle in which it spends part of the time in an apparently symbiotic, or benign, relationship in the gut of entomopathogenic nematodes from the family Heterorhabditidae (6) and part of the time killing and bioconverting the insect host which the nematode partner penetrates (5, 9). In this lifecycle, *P. luminescens* needs to be able to both kill its insect host and also to persist within the gut of its nematode carrier. This bi-phasic lifestyle presumably involves a switch between an insect-pathogenic and a nematode-symbiotic state. Several anti-insect virulence factors have been identified or inferred from biochemical and genetic studies (5, 8, 9). However available sample sequence from the *P. luminescens* genome shows that this bacterium may carry a number of different anti-insect toxins (7, 10), inplying either that these multiple toxins act at different sites and/or with different modes of action, or that *P. luminescens* uses functional redundancy or "overkill." Given that death of the insect host is critical to the successful release of new infective juvenile nematodes, which transmit the bacteria from host to host, this latter possibility is not unlikely.

To identify novel virulence factors in the *P. luminescens* genome, we have developed the tobacco hornworm caterpillar, *Manduca sexta*, as a model insect for bacterial infection (11). As an insect host, *M. sexta* has the advantages of both a large size and a well-studied insect immune system. We constructed a *P. luminescens* W14 cosmid library in *E. coli* and screened for insect virulence factors by injection of individual cosmids into *M. sexta* larvae. Normally, injection of wild-type *E. coli* into *M. sexta* results in rapid encapsulation of all of the bacteria by the insect hemocytes, completely clearing the infection from the hemocoel (11). These capsules, or nodules, are formed by complex interactions between different subpopulations of the hemocytes, which result in encapsulation of the bacteria and final melanization of the resulting capsule (12, 13). Once encapsulated the trapped bacteria subsequently die. Survival of *E. coli* within the insect hemocoel, in the face of an active immune system, is therefore unprecedented.

Here we report the isolation of a 33-kb *P. luminescens* cosmid that not only allows *E. coli* to persist within the insect but also results in a characteristic loss of body turgor followed by insect death. Insertional mutagenesis of the cosmid shows that a single, 8.8-kb gene, *makes caterpillars floppy* (*mcf*), is associated both with bacterial persistence in the host and also insect death. The amino acid sequence predicted by *mcf* shows only partial homology to known proteins; however, it does carry a BH3 domain, a domain found in pro-apoptotic proteins (14). The Mcf toxin appears to cause apoptosis in both the insect hemocytes and the insect midgut epithelium. The potential use of toxins active on both the midgut and the insect immune system is discussed.

Materials and Methods

Strains and Library Preparation. The cosmid library was prepared from *P. luminescens* subsp. *akhurstii* strain W14 genomic DNA by MWG Biotech (Munich). DNA was physically sheared, size selected for fragments of ≈30 kb, and then cloned into pWEB. The average insert size (≈32 kb) was determined by restriction analysis of a random selection of clones. For Southern analysis of *mcf* homologs, three other strains were used: *P. luminescens* subsp. *temperata* strain K122, *P. luminescens* subsp. *laumondii* strain TTO1, and *P. asymbiotica* [a new species recently split from *P. luminescens* (15)] strain ATCC43949. The first two strains were isolated from their nematode symbionts from Ireland and Trinidad, respectively. The third strain was isolated

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Abbreviations: GIM, Graces insect medium; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; GFP, green fluorescent protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF503504).

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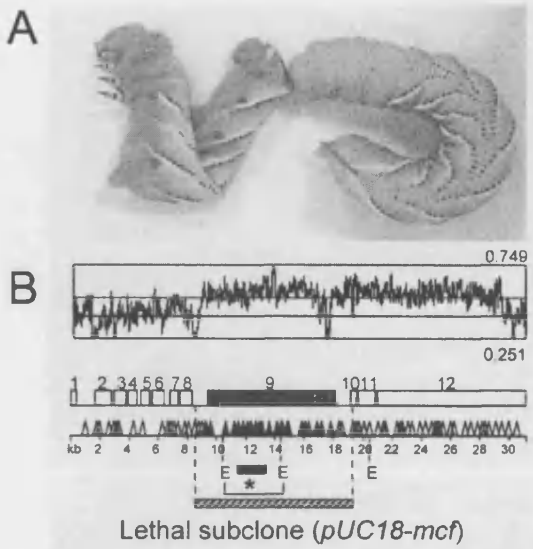

Fig. 1. The *mcf* gene causes loss of insect body turgor and is encoded within a putative pathogenicity island. (*A*) Fifth instar *M. sexta* larvae 24 h after injection of 2×10^6 *E. coli* cells containing H3 cosmid (left) and 2×10^6 *E. coli* cells without H3 cosmid (right). Note the loss of body turgor, or floppy phenotype, in the caterpillar on the left. (*B*) Genomic location and insertional mutagenesis of the H3 cosmid. The 33 kb of the H3 cosmid predicts 12 ORFs (ORF numbers above open boxes). Transposon mutagenesis shows location of transposons removing floppy and lethal phenotypes (▲) or leaving both phenotypes intact (△). The cluster of transposons that remove both phenotypes all lie within ORF nine, termed *mcf*. The relative location of a subclone containing only the *mcf* ORF is shown (E = *Eco*RI restriction sites). The shift in GC content (from 0.41 for the genome to 0.53 for the whole cosmid) across the region suggests that *mcf* lies within a pathogenicity island (see text). *, Probe used in the Southern analysis (see Fig. 2).

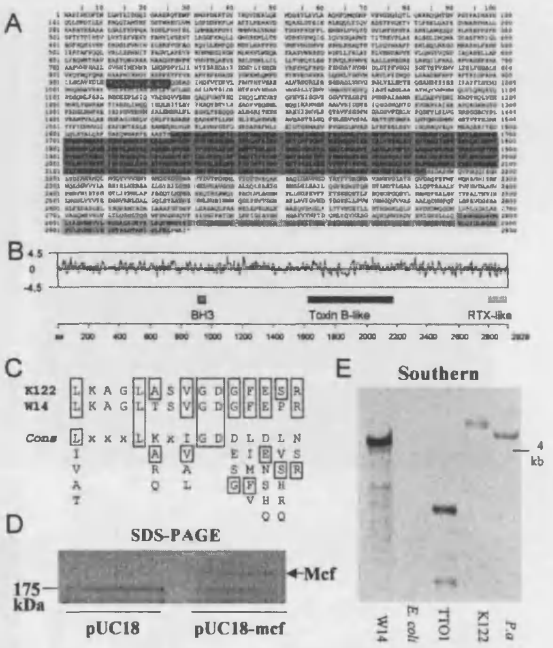

Fig. 2. The *mcf* gene predicts a novel toxin of high molecular weight. (*A*) Predicted amino acid sequence of the Mcf protein. The three shaded regions show regions of homology to other known proteins (see text). (*B*) Relative locations of the three regions of homology in relation to a Kyte-Doolittle hydrophilicity plot of the predicted protein. Note that the region homologous to the translocation domain of Toxin B is predominantly hydrophobic. (*C*) Match to the BH3 domain consensus sequence for Mcf toxins from two different *Photorhabdus* strains, W14 and K122. The consensus sequence for the BH3 motif is shown below. (*D*) SDS/PAGE gel of cytosolic fraction of Mcf expressing *E. coli*. An arrow indicates the presence of the Mcf protein (predicted weight of 324 kDa), which migrates at a higher molecular weight than the 174-kDa marker. (*E*) Southern blot of genomic DNA cut with *Eco*RI from four different *Photorhabdus* strains and *E. coli* probed with a fragment of *mcf* (see Fig. 1*B* for location of probe used). Note that the *mcf* DNA probe hybridizes at high stringency to all of the *Photorhabdus* strains but not *E. coli* (see text). Key to species and subspecies: *P. luminescens* subsp. *akhurstii* W14 (W14). *P. luminescens* subsp. *laumondii* TT 01(TT01), *P. luminescens* strain K122, which is probably subsp. *temperata* based on a description of its nematode host (K122), and *P. asymbiotica* ATCC43949 (*P.a*).

from a human wound, in the apparent absence of any potential nematode partner.

Insect Injection and Library Screening. The W14 cosmid library was arrayed into 96-well microtitre plates. For injection, individual clones were grown at 37°C overnight in 1 ml of LB with shaking. Ten microliters ($\approx 2 \times 10^7$ recombinant *E. coli* cells) of each bacterial culture was then injected into the hemocoel of individual fifth instar *M. sexta*. Injected larvae were scored for mortality after 48 h, in reference to control larvae injected with *E. coli* alone. In the first screen, injected larvae were examined at 24 and 48 h after injection. Clones showing insect mortality were rescreened three times, by injecting three individual larvae per clone, to look for a repeatable effect. Animals injected with positive clones also were examined for changes in appearance before death, such as changes in color or body turgor.

Cosmid Mutagenesis, Sequencing, and Southern Analysis. To ascertain which ORF within an individual cosmid was associated with the positive phenotype, we used insertional mutagenesis with a EZ::TN<TET1> transposon (Epicentre). Transposon mutants of individual cosmids were then re-screened to look for loss or retention of the phenotype (in this case loss of body turgor and death). Transposon mutants also were used as entry points in nucleotide sequencing of the positive H3 cosmid. This enabled us to ascribe loss of a specific phenotype to a specific gene. Cosmid DNA was prepared on a RoboPrep plasmid preparation robot (MWG Biotech) and sequenced on an ABI3700 nucleotide sequencer (Applied Biosystems). Sequences were assembled by using the LASERGENE software package (DNASTAR, Madison, WI). Southern analysis of *mcf* homologs in different *Photorhabdus* strains was performed by using a 4-kb *Eco*RI restriction fragment (Fig. 1) from W14 *mcf*.

Insect Histopathology and Hemocytes. For insect histopathology, whole larvae were fixed overnight in Bouin's solution (Sigma), after several incisions had been made in the cuticle to allow the fixative to permeate the cadaver. Larvae were then embedded in paraffin by using a Leica TP1020 automatic tissue processor. Sections were cut at 3–5 μm, stained with hematoxylin/eosin, and mounted on glass slides with DePeX-mounting medium. For terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) straining, similar larval sections were fixed in 4% paraformaldehyde in PBS and embedded in paraffin as described previously, using the TUNEL method according to the manufacturer instructions (*in situ* cell death detection kit, Roche Molecular Biochemical).

Hemocyte Monolayers. *M. sexta* larvae were selected 1 d after ecdysis to the fifth larval stage (instar). Insects were chilled on

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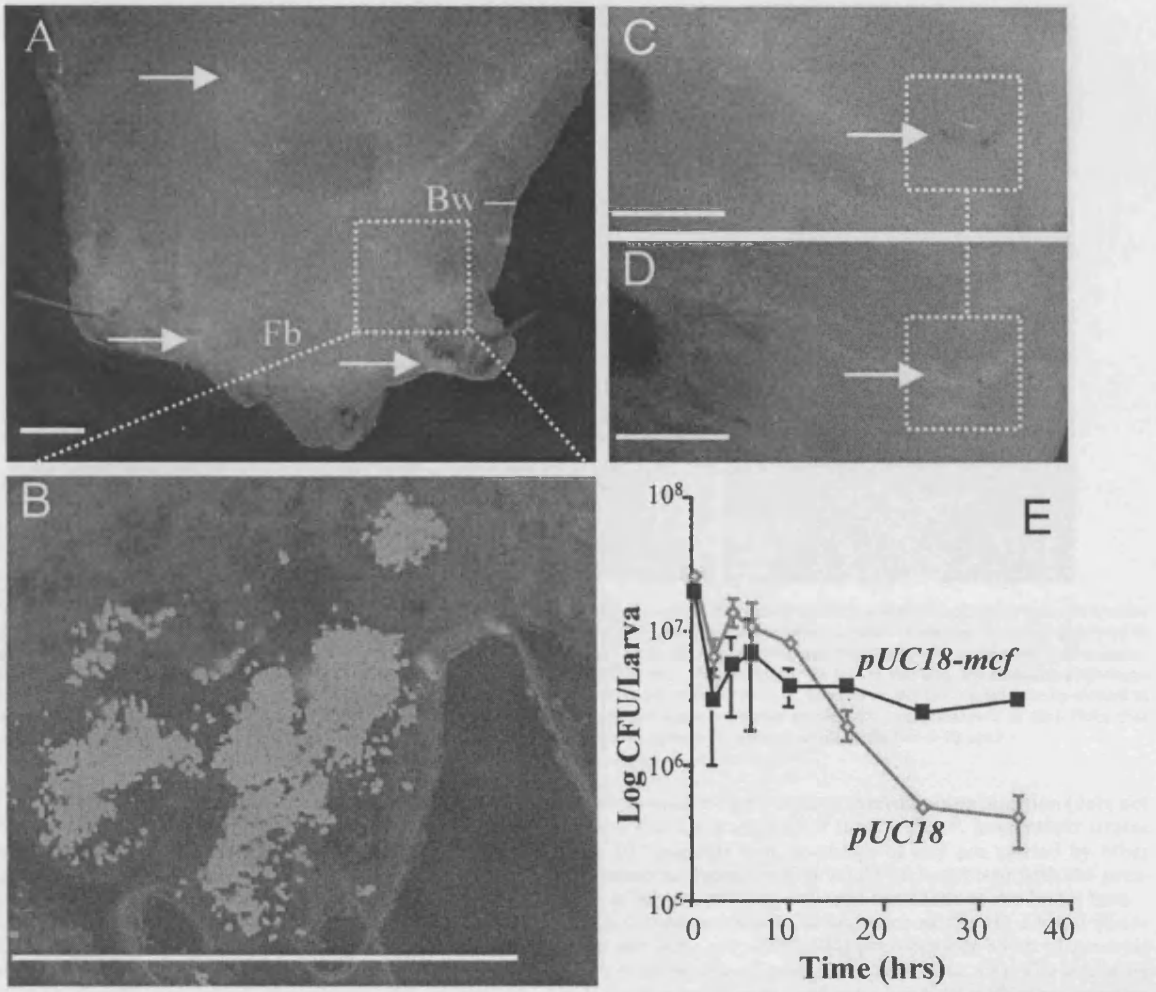

Fig. 3. GFP expressing *E. coli* carrying the *mcf* encoding subclone (*pUC18-mcf*) can persist within the insect model *M. sexta*. (*A*) Dissection of posterior of fifth instar larvae of *M. sexta* 16 h after injection of *pUC18-mcf*. Note the aggregations of GFP expressing bacteria within the fat body (Fb) and body wall (Bw) of the caterpillar. (*B*) High magnification of GFP-expressing bacterial clusters confirm that they consist of clumps of individual recombinant *E. coli*. (*C* and *D*) Parallel low magnification views of bacterial aggregations viewed under light and GFP-enhanced microscopy. Note how one of the bacterial aggregations (*C*) has been encapsulated by the insect hemocytes and brown melanin (arrow) deposited. A GFP enhanced view (*D*) of the same aggregations (arrows) shows the presence of GFP expressing and *pUC18-mcf* carrying *E. coli*. (Scale bar = 0.2 cm in *A–E*.) (*E*) Numbers of recoverable bacteria persisting over time in *M. sexta*. Note that *E. coli* carrying *pUC18-mcf* persist longer in an infected insect than *E. coli* carrying the *pUC18* vector alone. *E. coli* carrying *pUC18* alone were encapsulated as expected (data not shown).

ice for 30 min and swabbed with 70% ethanol before bleeding from the cut dorsal horn. Approximately 100 μl of hemolymph was allowed to drip into 900 μl of ice-cold Graces insect medium (GIM, Sigma) in a microcentrifuge tube. Hemocyte density was adjusted to $\approx 5 \times 10^6$ ml^{-1} in GIM by using a hemocytometer. Coverslips (10 mm) were washed in 70% ethanol and placed centrally into each well of a 24-well plate. The cell suspension (100 μl) was pipetted onto each coverslip and then left undisturbed for 60 min (room temperature) to allow the hemocytes to settle and form a monolayer. The monolayer was washed gently with a few drops of GIM and removed to a new well containing 400 μl of fresh GIM. Monolayers incubated with cytosolic fractions of Mcf expressing *E. coli* diluted 1:10 in GIM. For differential interference contrast microscopy imaging, a X40 Fluor water immersion lens was used on an upright Nikon E1000 microscope. Images were collected at 3-min intervals for 6 h with a Hamamatsu C4880–07 camera controlled by using METAMORPH software (Universal Imaging, Media, PA).

Results and Discussion

P. luminescens appears to encode numerous putative (7) and proven (8, 10) toxins in its genome. Although we understand the histopathological effects of some of these insecticidal toxins, such as the gut active Toxin complex A (16), we do not understand the role of specific toxins in insect death. To look for toxins capable of killing insects when expressed in recombinant *E. coli*, we screened a W14 cosmid library. After injection of 300 individual clones, we isolated a single cosmid (termed H3), which not only allows its *E. coli* host to persist within but also to kill its insect host. Injection of *E. coli* containing the H3 cosmid results in a rapid (within 12 h) loss of caterpillar body turgor (Fig. 1*A*) and death of the larvae (time of death, 24.1 h $\pm$ SE of 3.25). We

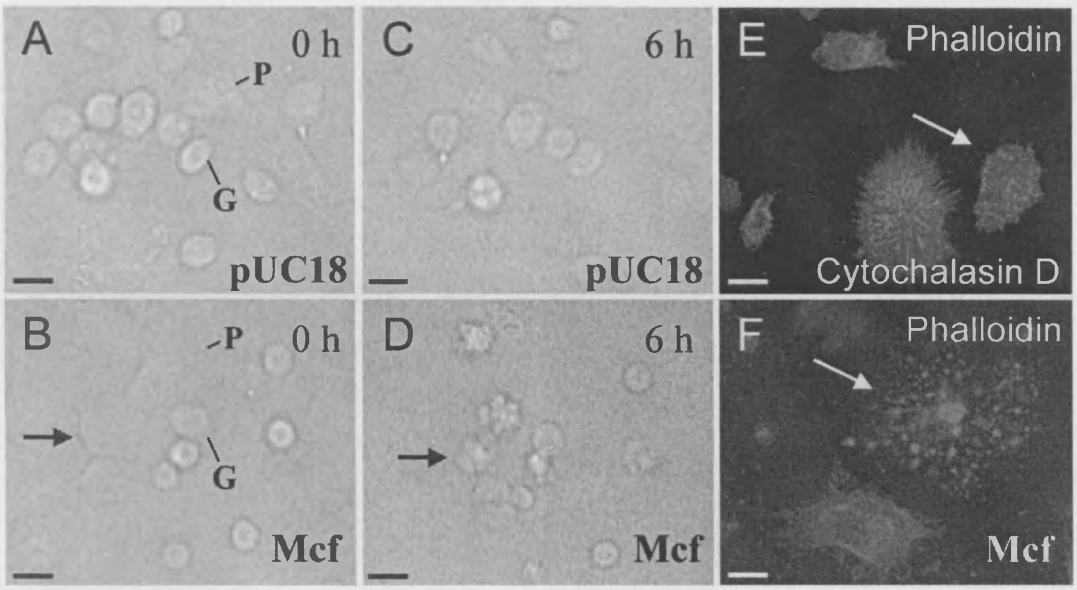

Fig. 4. Hemocyte monolayers show that Mcf toxin acts on insect blood cells within 6 h by disrupting the cytoskeleton. (*A* and *B*) Hemocyte monolayers treated with cytosolic fractions of either *pUC18* or *pUC18-mcf* carrying *E. coli* at the start of time-lapse differential interference contrast microscopy imaging. (*C* and *D*) The same preparations 6 h after treatment. Note that the plasmatocyte (P) indicated by an arrow (*D*) has begun to disintegrate by producing a series of rounded blebs. Note that the cell highlighted by an arrow is the same cell (*B* and *D*). Taken together with observations from TUNEL staining, the blebbing phenotype associated with Mcf-mediated hemocyte death is strongly supportive of programmed cell death. The full time lapse series of Mcf toxin action can be viewed at www.bath.ac.uk/bio-sci/quicktime.htm. (*E* and *F*) FITC-phalloidin staining of hemocyte monolayers treated either with cytochalasin D or Mcf. Note that treatment with both toxins disintegrates the actin cytoskeleton leaving a punctate staining pattern of residual actin. (Scale bar = 10 μm.)

termed this loss of body turgor the "floppy" phenotype. Insertional mutagenesis of this cosmid (Fig. 1*B*) shows that a single ORF, termed *mcf*, is responsible for these phenotypes. Inserted transposons also were used as entry points for sequencing of the entire cosmid. This dual approach allowed us both to derive nucleotide sequence of the entire 33-kb cosmid and to attribute phenotype loss to a specific gene. Sequencing of the cosmid was completed from 126 transposon entry points, 39 of which negated both floppy and lethal phenotypes. All of the 39 insertions associated with phenotype loss lie within the 8.8-kb predicted ORF of *mcf* (Fig. 1*B*). To confirm the hypothesis that *mcf* causes both the floppy and lethal phenotypes associated with the complete cosmid, we subcloned the *mcf* gene alone (Fig. 1*B*). This subclone also confers the same ability on *E. coli* to both render caterpillars floppy and to kill them within an equivalent space of time (data not shown).

The *mcf* ORF predicts a high molecular weight protein of 2,929 aa (Fig. 2*A*) with a molecular weight of 324 kDa and PI of 5.4. Searches of current databases reveal three areas of similarity with known proteins (Fig. 2*B*). First, Mcf contains a consensus sequence for a BH3 domain (Fig. 2*C*). Proteins involved in apoptosis that contain only this domain are pro-apoptotic (14). Second, residues 1,626–2,139 show 20% identity to residues 873–1,362 from *Clostridium difficile* toxin B. This region lies within part of toxin B thought to be involved in translocation (17). Finally, the predicted C terminus of Mcf contains a region (residues 2,791–2,925) that shows similarity to a repeated sequence in the C terminus of *apxIVA*, an RTX-like toxin from *Actinobacillus pleuropneumoniae* (18). The remainder of the predicted protein shows no significant homology to known proteins and little variation in predicted hydrophobicity (Fig. 2*B*). SDS/PAGE analysis of cytosolic preparations of *mcf* encoding recombinant *E. coli* reveal the presence of a single protein of the predicted molecular weight (Fig. 2*D*). These preparations are highly toxic to caterpillars on injection (data not shown). Southern analysis of three other *P. luminescens* strains (Fig. 2*E*) suggests that homologs of *mcf* are carried by other *Photorhabdus* strains, which would be consistent with the presence of the toxin being required for death of the insect host.

The complete nucleotide sequence of the H3 cosmid (GenBank accession no. AF503504) encompasses 33 kb of genomic DNA and contains 12 predicted ORFs (Fig. 1*B*). The similarity of the surrounding predicted gene products with other putative virulence factors and the shift in GC content (Fig. 1*B*) associated with the genomic location of *mcf* suggests that this region constitutes a pathogenicity island. Thus, upstream of *mcf* is an ORF (orf 4), which shows similarity to *dlpA* or *dotA* of *Legionella pneumophila* (19). The *dot/icm* system encodes a type IV secretion system related to the *Shigella flexneri* Col1b Incl plasmid (19). Within this system, DotA encodes a cytoplasmic membrane protein required for macrophage cell killing by *L. pneumophila* (20). Downstream of *mcf* lie two ORFs (orfs 11 and 12), which predict proteins with similarity to the HecA and HecB hemolysins also found in a wide range of other Enterobacteriaceae. The presence of these macrophage killing and hemolysin-like genes suggests that this pathogenicity island may encode several genes involved in overcoming host blood cells.

To investigate how *E. coli* carrying the *mcf* containing *pUC18* plasmid (*pUC18-mcf*) can persist within *M. sexta*, we labeled *pUC18-mcf* with a second plasmid (*pBBR2*) expressing green fluorescent protein (GFP). We then injected caterpillars with the marked *pUC18-mcf* and dissected caterpillars at different time intervals postinjection to look for *E. coli* expressing GFP. Dissection of *Manduca* infected with the GFP expressing *mcf E. coli* showed visible aggregations of bacteria persisting within insect tissues (Fig. 3 *A* and *B*). Quantitation of bacterial persistence over time (Fig. 3*E*) shows that *pUC18-mcf* containing *E. coli* can persist longer in the presence of the insect immune

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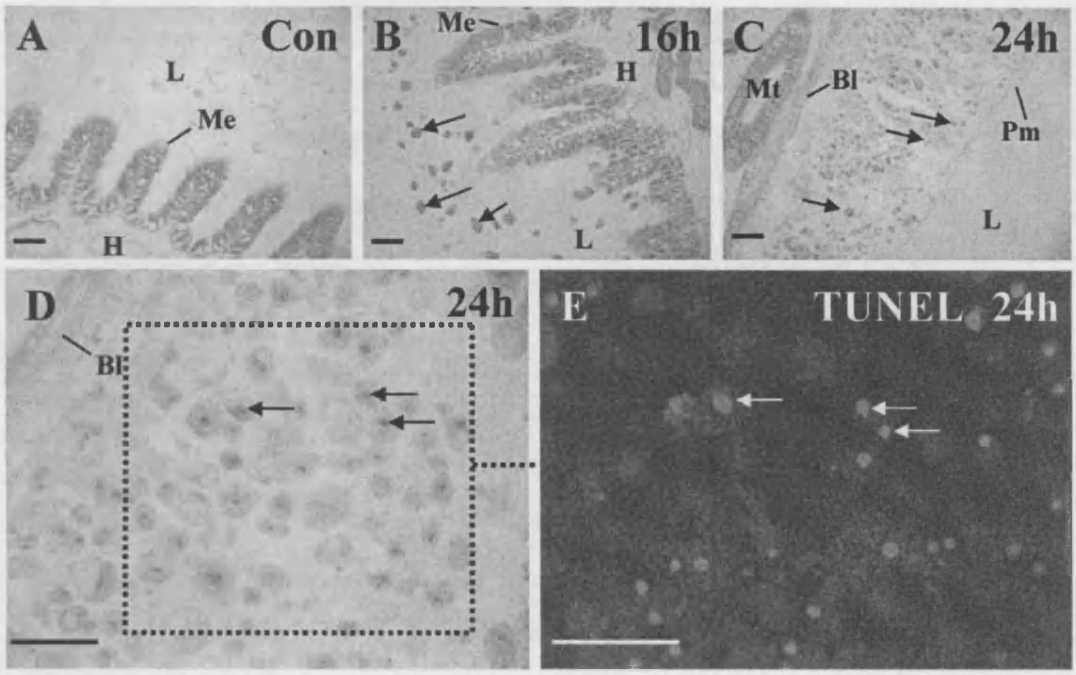

Fig. 5. *M. sexta* infected with Mcf encoding *E. coli* show massive destruction of the midgut epithelium. (*A*) Light microscopy of a stained control section of *M. sexta* fourth instar midgut. Note the midgut epithelium (Me), which separates the lumen (L) of the gut from the insect hemocoel (H). (*B*) Section of midgut 16 h after infection with *pUC18-mcf E. coli*. The cells of the midgut have begun to bleb into the gut lumen (arrows). (*C*) Section of midgut 24 h after infection. The midgut epithelium has disintegrated leaving the space between the basal lamina (Bl) and the peritrophic membrane (Pm) packed with blebbed cells. (*D*) Detail of disintegrated epithelium showing that nuceli within the cellular blebs are pycnotic and often surrounded by vacuoles. (*E*) TUNEL staining of disintegrated epithelium suggesting that the cells are undergoing apoptosis. (Scale bar = 100 μm.)

system than *E. coli* containing *pUC18* plasmid alone. *E. coli* carrying *pUC18* alone were encapsulated at high frequency as previously documented by others (21). Detailed examination of the bacterial aggregations often shows partial encapsulation and melanization of the bacteria (Fig. 3 *C* and *D*). These observations suggest that although the Mcf expressing *E. coli* are recognized by the hemocytes, they cannot be successfully encapsulated. Such a phenotype could be caused by the direct toxicity of expressed Mcf on hemocytes attempting to adhere to and encapsulate the bacteria or by interference with the melanization cascade.

To test the effect of Mcf on hemocytes, we added preparations of Mcf protein to hemocyte monolayers and examined them under differential interference contrast microscopy illumination and time-lapse photography (Fig. 4). In the presence of cytosolic fractions from *E. coli* carrying *pUC18* alone, hemocytes exhibit normal motility and can persist for at least 12 h as a monolayer with no visible adverse effects (Fig. 4 *A* and *C*). Granulocytes show limited motility whereas the more amoeboid plasmatocytes move freely across the monolayer (www.bath.ac.uk/bio-sci/quicktime.htm). In the presence of all fractions containing Mcf, both cell types show marked changes in morphology within 6 h and affected cells begin to disintegrate by producing numerous circular blebs (Fig. 4*E*). This phenotype is similar to that observed in other cell types undergoing apoptosis (22). Taken together with the observations from TUNEL staining, the blebbing phenotype associated with Mcf-mediated hemocyte death is consistent with Mcf triggering programmed cell death. Actin straining of Mcf-treated cells shows that the cytoskeleton has disintegrated, remaining only as a series of highly staining foci (Fig. 4*F*). We note that this final actin staining pattern is similar to that achieved by treatment of hemocytes with the fungal toxin cytochalasin D (Fig. 4*E*) (23).

Finally, to examine the potential cause of the rapid loss of body turgor associated with the floppy phenotype, we examined the histopathology of insect hosts infected with *mcf* containing *E. coli*. Sections of infected larvae were stained and examined via light microscopy (Fig. 5 *A–D*). Given that both the insect midgut and the malpighian tubules are associated with osmoregulation of the insect hemolymph, disruption of either of which could cause the floppy phenotype, we examined the histopathology of these structures in detail. The malpighian tubules (approximating in function to vertebrate kidneys) appear unaffected before the time of insect death, whereas the midgut epithelium is severely affected as early as 12 h. The midgut epithelium is a simple epithelium composed of two main cell types (columnar and goblet cells) and is fringed on the lumen side with a brush border membrane (Fig. 5*A*). After infection with *mcf* containing *E. coli*, the midgut epithelium begins to bleb into the lumen of the gut. Both goblet and columnar cells elongate and shed circular blebs often containing the cell nucleus (Fig. 5 *B–D*). These nuclei appear pycnotic, are often surrounded by a perinuclear vacuole, and stain TUNEL positive (Fig. 5*E*), suggesting that they are apoptotic. As the midgut is one of the primary organs responsible for osmoregulation, such systematic destruction of the midgut epithelium would be consistent with the loss of body turgor associated with the floppy phenotype.

In conclusion, the gene *mcf* encodes a novel high molecular weight toxin that destroys both the insect gut and insect hemocytes. As both affected tissues show clear signs of apoptosis, these data are consistent with the hypothesis that *mcf* encodes a novel BH3 containing pro-apoptotic toxin. Destruction of the

hemocytes during infection correlates with a failure to successfully encapsulate and clear Mcf expressing bacteria during infection. Destruction of the insect midgut, a primary organ in osmoregulation, is probably responsible for the marked loss of body turgor observed in the floppy phenotype. As both the hemocytes and the midgut are destroyed by injection of either *E. coli* expressing Mcf or by Mcf toxin itself, we can infer that Mcf interacts with, or is uptaken by, the hemocoel side of the insect midgut. However, the normal method of secretion of Mcf from *P. luminescens* itself, or its mode of action on insect cells, remains obscure. Finally, although there is considerable current interest in toxins which act on the insect gut, such as the δ-endotoxins from *Bacillus thuringiensis* (24) and the toxin complexes from *P. luminescens* (8), toxins, like Mcf, which act on both the gut and the insect immune system represent a promising, yet underexploited, avenue for future insecticide development.

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